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THE INFLUENCE OF IRON ON PIGMENTATION AND
RESISTANCE TO IONIZING RADIATION IN THREE STRAINS
OF MICROCOCCUS VIOLAGABRIELLAE

by

Morley Bleviss

A THESIS

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The undersigned certify that they have read,
and recommend to the Faculty of Graduate Studies for
acceptance, a thesis entitled "The Influence of Iron on
Pigmentation and Resistance to Ionizing Radiation in
Three Strains of Micrococcus violagabriellae" submitted
by Morley Bleviss in partial fulfilment of the require-
ments for the degree of Master of Science.

ABSTRACT

In the present work three strains of Micrococcus violagabriellae (Littlepig, Pig (Parental) and Superpig) were used. The pigment, (which imparts a violet-red color to the colonies of the Parental- and Superpig strains), is only produced when the cells are grown in the presence of excess iron. We are thus presented with a unique system for the study of the radio-protection conferred by a pigment.

Cell suspensions of each of the three strains were made from organisms grown in the presence and absence of excess iron. These were exposed to gamma rays and plated on media containing an excess of iron and on media without iron. Evidence is presented correlating the color intensity of the pigment (pulcherrimin), and the ability of the organism to survive moderate doses of ionizing radiation.

Studies using radioactive iron (^{59}Fe) showed that a correlation also exists between the color and the amount of iron that the cells of each strain can remove from the medium. Various cell regulatory systems are suggested as a possible mechanism of control over pigment synthesis.

The effect of γ -irradiation of the cells, on the post-irradiation growth by the organisms is discussed for each strain irradiated during logarithmic growth and during a stationary state.

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INTRODUCTION

Moseley and Laser (1965a) state:

"The radiosensitivity of different vegetative bacteria varies to a very great extent. For example, the dose of ionizing radiation of X-rays or ^{60}Co gamma rays required to reduce the viability of Pseudomonas by 50% (LD_{50}) is less than two krads., while a dose of 100 krads. is required to produce the same effect in Micrococcus radiodurans. The latter bacterium therefore possesses a 500 - fold greater radioresistance at this level of survival" (Page 210).

Studies to determine the radioprotective ability conferred on a bacterium by a pigment, have of necessity been comparisons between pigmented wild types and non-pigmented mutants. For example, Duggan, Anderson, and Elliker (1963) reported that a decrease in pigmentation in their mutant strain Rw of M. radiodurans was correlated with an increase in gamma radiation sensitivity. Moseley and Laser (1965a) obtained similar results in the same organism using their non-pigmented mutants W_1 and W_2 . In these comparisons genetic events which govern functions other than pigment production may also be involved.

The Micrococcus violagabriellae system used in the present experiments is unique, in that it allows a study of the radio-resistance conferred by a pigment under conditions of genetic and/or environmental change. In two of the three known strains of this organism (Payne and Campbell, 1965b), the

presence of ferric and/or ferrous ions in the growth media induces the production of a reddish-violet pigment in an otherwise white-pigmented bacterium. The third strain however remains white in the presence of iron. We thus have a system in which the presence or absence of a single environmental factor (namely iron) may produce a detectable change in the expression of a color trait. Consequently, the use of such a system allows an estimate of the contribution by a single genetic trait to the response of the organism to an adverse environment (e. g. ionizing radiation).

LITERATURE REVIEW

The Organism

Micrococcus violagabriellae was named by Castellani, (1956) and isolated from an erythematous disease of the axilla of American sailors, who had apparently contracted the infection in the West Indies and Central America. Although the organism has been classified as a Micrococcus, more recent information (Auletta and Kennedy, 1966; Rosypal, Rosypalová and Horejš, 1966) suggests the possibility that it may belong to the Staphylococcus group (Castellani in a later publication, (Castellani, 1957) termed the organism Staphylococcus violagabriellae). This controversy has been dealt with at some length by Baird - Parker (1963 and 1965). According to this latter author the criterion for the classification of this organism is based on the ability of the Staphylococci to grow and produce acid from glucose anaerobically. He states: "The staphylococci are a group of potential pathogens or commensals which are characteristic of animal and human surfaces. The micrococci are a group of mainly free - living saprophytes which are nutritionally less exacting and morphologically and biochemically more variable than the staphylococci" (Page 376). In addition, Jones, Deibel and Niven (1963) consider the organism to be a Staphylococcus whereas Baird - Parker (1965) put M. violagabriellae as belonging in subgroup II in his classification system. Kocur and Martinec (1963) on the other hand consider M. violagabriellae

to be a strain of Staphylococcus epidermidis. More recent work (Jones et al., 1963; Steel, 1964; Baird - Parker, 1965; Auletta and Kennedy, 1966; Rosypal et al., 1966) suggests that although the organism is closely related biochemically to Staphylococcus epidermidis, the designation Staphylococcus violagabriellae would be more appropriate. Auletta and Kennedy (1966) suggest the name Staphylococcus violagabriellae on the basis of the percentage of guanine and cytosine(G-C) present in the DNA. They found that Staphylococci had a 30-39% G-C content while Micrococci had 59 - 69% G-C. M. violagabriellae was found to have 30% G-C content.

The Pigments of *M. violagabriellae*

The first suggestion that the pigment of the yeast *Candida pulcherrima* (pulcherrimin) was similar to the violet-red pigment of *M. violagabriellae* came from Canale - Parola (1963). The yeast pigment had previously been isolated by Kluyver, Van Der Walt and Triet (1953) and attempts have since been made to characterize the bacterial pigment as well (Campbell, Nichols and Berry, 1964; and MacDonald, 1966). According to these latter authors the compound is the ferric salt of pulcherrimic acid. Kluyver et al. (1953) and Cook and Slater (1954 and 1956) have proposed probable structures for pulcherriminic acid, the most likely of which appears to be that shown in Figure 1, proposed by Cook and Slater (1956) and later confirmed by MacDonald (1962). The structure of its derivative "pulcherrimin" as proposed by Cook and Slater (1956) is given in Figure 2.

Campbell et al. (1964) postulated that the Fe-dependent pigment in *M. violagabriellae* is an iron chelate of pulcherriminic acid with an associated low molecular weight peptide moiety, with glycine as the sole N-terminal amino acid. MacDonald (1966) found that the proton magnetic resonance spectrum of the free acid of *M. violagabriellae* was identical to that of free pulcherriminic acid obtained from *C. pulcherrima*.

Campbell et al. (1964) state that the free acid of *M. violagabriellae* is similar to that of *C. pulcherrima*; the pigments themselves however, differ

in that the peptide moiety is associated with the pigment in M. violagabriellae only. Another difference appears to be the way in which iron is either bound or used. It was found, for example, that the Micrococcus pigment could be reprecipitated from an alkaline solution (which removes Fe as $\text{Fe}(\text{OH})_3$) without re-introducing additional iron. This, however, is not true of the yeast (C. pulcherrima) pigment (Campbell et al., 1964; MacDonald 1965).

Yet another difference appears in the method of pigment production. The biosynthetic pathway in the yeast as proposed by Miller (1961) and MacDonald (1965) appears to be quite different from that suggested by Campbell et al. (1964) for M. violagabriellae. In the latter, the aerobic cytochrome-linked electron-transport system appears to be involved. The Miller and MacDonald pathway however, involves the combination of two leucine molecules to produce a single cyclo-leucyl-leucyl molecule which becomes pulcherrimic acid. (Figure 3)

A second pigment found in M. violagabriellae by Nichols and Campbell (1964) was a melanin-like brown pigment which required iron for formation, although iron was not incorporated into the pigment itself. This pigment was only produced in the presence of 0.01M cresol. These authors proposed that this new pigment was an alternative end product of normal pigment synthesis. This proposal was based on the observation that the requirements for formation of normal melanins in Pseudomonas species were different from those in M. violagabriellae. For example, the melanin

precursor, dopa, B-(3, 4 dihydroxy phenyl) L-alanine, could not be found in M. violagabriellae, and when it was added to the cells or their extracts, no additional pigment was produced. Furthermore, M. violagabriellae was not dependent upon tyrosine for formation of the pigment, while Pseudomonas species required it.

Nichols and Campbell (1964) suggest that cresol probably blocks some step in the normal biosynthetic pathway of pigmentation resulting in the production of an alternative end product. Such a block would occur after the iron - dependent step or steps, and before iron is incorporated into the pigment.

Figure 1. The structure of pulcherriminic acid
according to Cook and Slater (From MacDonald, 1962).

Figure 2. The structure of pulcherrimin according
to Cook and Slater (1956).

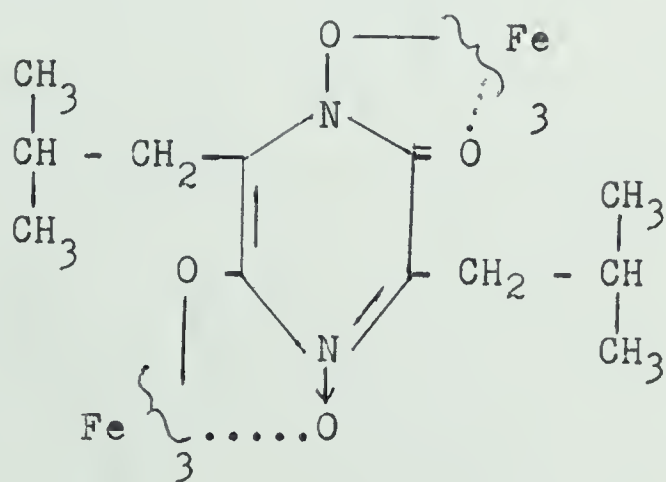
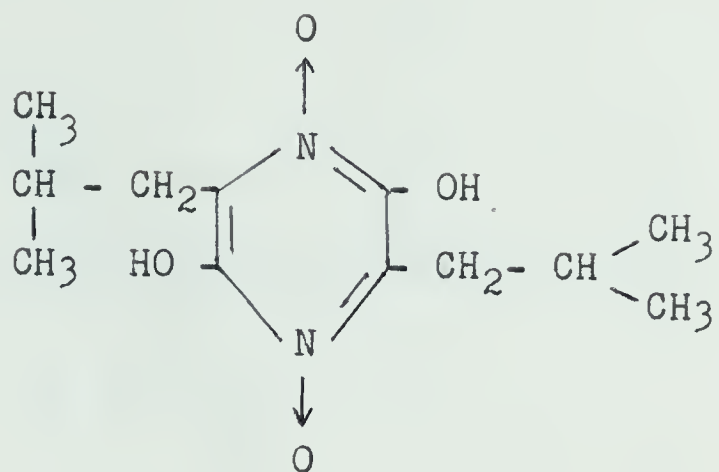
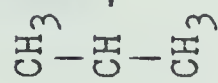
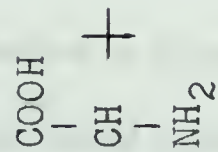


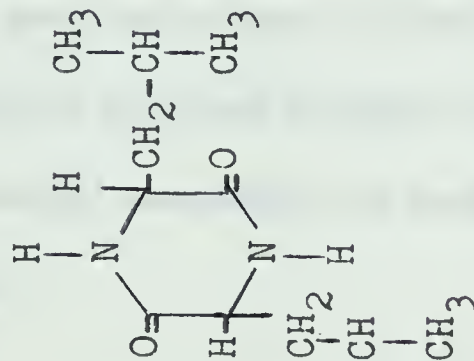
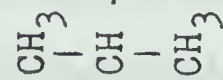
Figure 3. The biosynthetic pathway of pulcherriminic acid in the yeast Candida pulcherrima (from MacDonald, 1965).



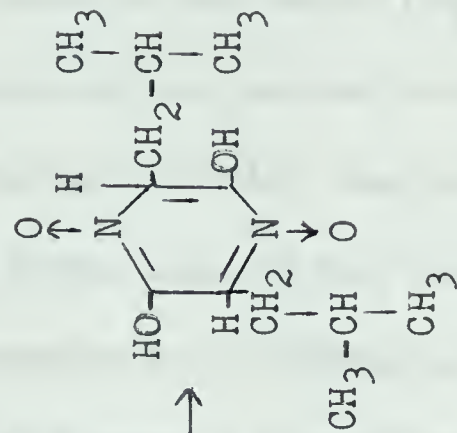
leucine



leucine



cyclolucyl-leucyl



pulcherriminic acid

Effects of Ultraviolet Light on *M. violagabriellae*

Payne and Campbell (1962 and 1965a) found that the presence of pigment in the cells of the Parental (Pig) strain, after growth on media containing an excess of iron, provided the cells with more than twice the normal resistance to ultraviolet light. They also found that the white-pigmented cells (those grown in the absence of iron) when exposed to ultraviolet light and plated on media containing iron, exhibited post-irradiation recovery. Furthermore, anaerobic cells (pigment is not produced under anaerobiosis (Payne and Campbell 1965a)) were more resistant to ultraviolet light than aerobic cells. Subsequent studies by Payne and Campbell (1963 and 1965a) showed that ultraviolet radiation (of wavelengths less than 300 m μ) was responsible for the lethal action.

In addition, these authors found that if NO₃ were present under anaerobic conditions, the normal sensitivity to ultraviolet radiation (i.e. sensitivity under aerobic conditions) was restored, and suggested that the site of the damage was extranuclear and related to the pigment synthesising mechanism, which in turn was associated with the cytochrome-linked electron transfer system. The NO₃ acted as an alternative to O₂ as the terminal electron acceptor. This protective mechanism was also found to be true for sources of iron which did not support pigment production (haemoglobin, dialysed haemoglobin, and haemin) (Payne and Campbell, 1965a).

In all cases, the protective effect was only noted for short term exposure to low doses. Payne and Campbell (1964a) proposed that at high doses the nuclear sites, which had not been affected by iron or the presence of O_2 , were irreversibly damaged.

Moseley and Laser (1965 b) have shown that there is evidence for a common mechanism, operative in the repair of lethal ionizing and ultraviolet radiation damage, in Micrococcus radiodurans. They were able to find a radiation resistant non-pigmented strain, (Moseley and Laser, 1965 a) and therefore suggested that the pigment did not act as an "energy sink", (as suggested by Kilburn, Bellamy, and Termi (1958)) but rather, that an enzymatic repair system similar to that of the ultraviolet dark-repair system, was operative.

Thornley, Horne and Glauert (1965) with the aid of electron microscopy have found that there is an extremely complex cell wall and sheath present in M. radiodurans which may act as an "energy sink" and reduce the effect of both electromagnetic and ionizing radiation.

Mechanism of Action of Ionizing Radiation on Biological Material

Direct Action

When an ionization occurs in a metabolically active organic molecule (e.g. in an enzyme or nucleic acid), the observed inactivation of the molecule is said to be due to the "direct action" of the ionization. This is in contrast to the "indirect action" of radiation which is of a secondary nature (Hutchinson and Pollard, 1961). When the direct action predominates, the "Target Theory" is used to interpret the results. This theory was expanded upon by Lea (1946) in his book: Actions of Radiations on Living Cells.

The important characteristic of ionizing radiation, according to Hutchinson's interpretation (1961) of the target theory, is the localized release of large amounts of energy which can break chemical bonds with relative ease. This disruption, if it occurs in a metabolically active compound (e.g. in an enzyme), would prevent the compound from carrying out its highly specific biological function. Lea (1946) has suggested and Pollard, Guild, Hutchinson and Setlow (1955) have confirmed, that in many cases a primary ionization within a protein molecule is sufficient to inactivate it biologically.

Whether a vital target is hit or not, is a statistical consideration varying directly with the volume of the target and the dose which is administered to it. The result of the direct action is an exponentially decreasing survival curve

with increasing dose. According to Hutchinson (1961), "The plot of the survivors of more complex organisms as a function of dose is usually sigmoid (if the dose is plotted on linear paper), with a shoulder at low doses and an exponential slope at high doses (if survivors are plotted on semi-logarithmic paper). This is interpreted as meaning that either multiple targets are present or multiple hits in the same target are needed for inactivation". Butler (1956) pointed out that "there is no reason to expect that a slight denaturation or even more drastic changes of enzymes which are present in quantity will have a drastic effect on the life of the cell". A vital target would therefore have to be uninducable, irreplaceable, irreparable, and present in only one or a few molecules, in order to cause cell death. For this reason DNA is considered to be the most likely target of the direct action (Errera, 1955). Studies of light scattering (Alexander and Stacey, 1956) and viscosity changes (Butler, 1959) indicate that the primary effect of ionizing radiation on DNA appears to be to split the molecule into fragments.

The available evidence, according to Kaplan (1963), suggests that, although there may be many molecules of DNA in a cell, there will be very few (perhaps only one) with a particular base sequence. Since DNA acts as a template for its own replication, damage to it would be irreparable, and its loss could be fatal to the cell.

Indirect Action

Several good reviews of the indirect action of ionizing radiation have

been published (see Barron, 1955; Dale, 1954; Grosch, 1965; Hart and Platzman, 1961; Hutchinson, 1961; Kaplan, 1963; Kelner, Bellamy, Stapleton, and Zelle, 1955; Powers, 1961; Schwarz, 1964; and Stein and Swallow, 1957). The prevalent opinion is that, because the bacterial cell is composed of 70 - 90% water, most of the ionizations occur in water, leading to the immediate production of highly reactive but short-lived free radicals, possessing unpaired electrons (Figure 4 (a)) (Grosch, 1965). These free radicals may recombine harmlessly to yield water again, or may interact with nearby macromolecules, usually causing chemical bonds to be broken. The consequences of the destruction of these macromolecules to the cell depends on (a) the number of intact molecules of the substance which remain; and (b) the capacity of the cell to repair the damaged molecules or to replace them with new ones. Again, as with the direct action, DNA is the most likely macromolecule to be affected in such a way as to be fatal to the cell.

Free radicals can react in a variety of ways with each other (Figure 4b and 4c) and with macromolecules in the cell, depending on the geometric distribution of the primary products (Grosch, 1965). For instance, the interference with the sub-cellular fine structure of mouse leukaemia cells as an important primary lesion leading to eventual cell death, was discussed by Alexander and Bacq (1961). These authors found that this non nuclear material cannot readily be repaired or replaced. However, they found no evidence for nor postulated any similar action occurring in bacterial cells.

It has been suggested (Allen, 1961) that the ionization products of water lead to the production of organic peroxides as a result of reactions involving the organic compounds found in abundance in the cell. These organic peroxides may also react with the more complex macromolecules to produce inactivation.

It is obvious that the inactivation of a protein or an RNA molecule, which can easily be replaced, or which is present in large amounts, would have no effect on the ability of the cell to remain alive. Therefore to be lethal to a cell the radiation must alter the DNA or, it must interfere with other material that cannot be easily repaired or replaced (Alexander and Bacq, 1961).

Figure 4.

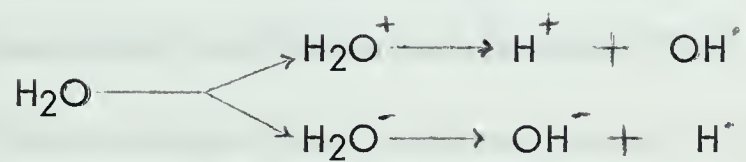
The formation and reactions of free radicals and their products.

(a) Free radical formation in water after irradiation (From Grosch, 1965).

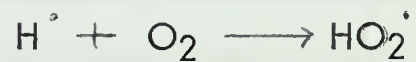
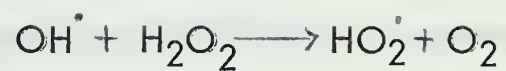
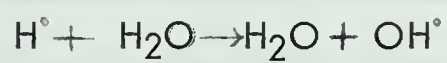
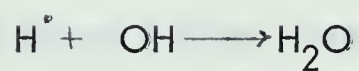
(b) Possible free radical reactions (From Grosch, 1965).

(c) Possible reactions of one of the products with a macromolecule (From Grosch, 1965).

(a)



(b)



(c)



Characteristics of Sensitive and Resistant Cells

Each microorganism is a complex unit which may possess characteristics not found in other organisms and it is therefore not surprising that the individual response to ionizing radiation differs from species to species. Terzi (1965) and Kaplan and Moses (1964) correlated genetic complexity of microorganisms with their radiosensitivity and Kaplan and Zavarine (1962) studied radiosensitivity in relation to the percentage of guanine plus cytosine (G-C) present in the genetic material. Adler (1966) in a subsequent contribution suggested that factors other than the percent G-C play a more important role in conveying radiosensitivity upon an organism and lists as examples the nucleotide sequences, the presence of energy transfer mechanisms, and gene-controlled repair phenomena.

Pontefract and Thatcher (1966) showed that a positive correlation exists between the length of the cells in an E. coli culture and their resistance to γ -irradiation. Elongated cells with increased numbers of nuclei, and the presence of a budding phenomenon, are characteristic of the more resistant cell cultures. The characteristic 'elongated cells' was not found to be a result of irradiation but rather to be a genetic trait, as the cells of both the parent culture and the radiation resistant culture were found to have identical DNA content.

The effect of oxygen on the response of bacterial cells to radiation has been studied in great detail. (See Hollaender, Stapleton and Martin, 1951;

Hollaender and Stapleton, 1953; Patt, 1953; and Zelle, 1955.) The presence of oxygen increases the sensitivity of cells: the oxygen reacting irreversibly with free radicals to form oxyradical or peroxyradical complexes, which are strongly oxidizing, and which can bring about secondary reactions damaging to the cell (Powers, 1961).

Stapleton (1955, and 1955, in Kelner et al.) correlated radiation sensitivity with the different stages in the growth cycle of E. coli (see Figure 5). He concluded that the period from the onset of the initial lag phase to just before cell division is characterized by an increased resistance to irradiation, whereas during the logarithmic phase a rapid decrease in resistance takes place, reaching a minimum at the end of this phase. As the cells move into the stationary phase a gradual return to the initial resistance can be observed.

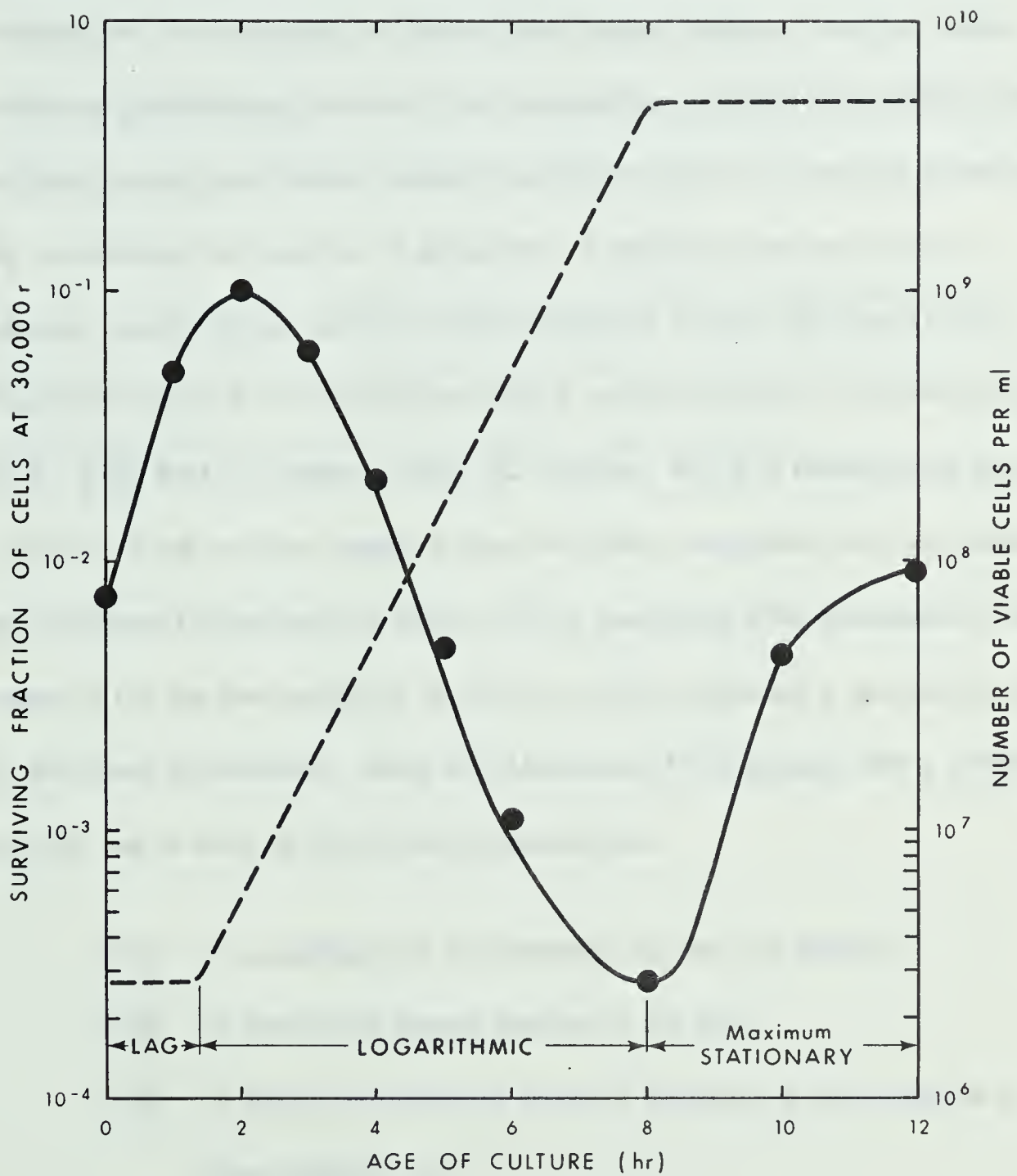
Stapleton (loc.cit.) was able to show that different growth media yield different survival curves and that the temperature of incubation before and after irradiation may play an important role in resistance. The work of Thornley et al. (1965) suggests that the cell wall and sheath structures present may modify the effect of radiation in Micrococcus radiodurans.

In conclusion, the inactivation of bacteria in general depends on: (a) the physiological state of the organism at the time of irradiation, (b) the presence or absence of elaborate physical barriers in the cell and (c) the

atmospheric and environmental conditions (e.g. temperature, O_2 concentration et cetera) under which they are irradiated.

Figure 5.

Survival of E. coli cells at a constant dose of X-rays as a function of the age of the culture. (From Stapleton, In: Kelner et al., 1955)



Protection of Bacteria from the Lethal Effects of Ionizing Radiation

Hollaender and Stapleton (1953) attribute the protection of E. coli cells against radiation afforded by some substances the organism is able to metabolize, to a decrease in intracellular oxygen tension. In other words, radiation protectors, by virtue of their metabolism, deplete the available intracellular oxygen and thereby reduce the indirect action of ionizing radiation by preventing the formation of peroxides. Examples given are alcohols, glucose, amino acids, and any of the carboxylic acids of the Krebs cycle. Further evidence for this is the fact that β -alanine (which is not metabolized by E. coli) does not protect, while α -alanine, which is metabolized does protect. These workers suggest further that other compounds such as cysteine and sulfhydryl substances act either, (1) by competing with radiosensitive cell material for the free oxidizing radicals or, (2) by replacing a metabolite that is destroyed by radiation. Bacq and Alexander (1955) suggest that a protector acts by one or more of the following mechanisms:

- (1) it competes with cell material for the free radical,
- (2) it lowers the oxygen tension in the cell,
- (3) it reacts with the cell material rendering it non-reactive to free radicals, or,
- (4) it may accept energy stored in a macromolecule after it receives an ionization.

Some compounds that are known protectors of E. coli are, (1) Sodium Hydrosulfite ($\text{Na}_2\text{S}_2\text{O}_4 \cdot 2\text{H}_2\text{O}$), which acts by reducing oxygen tension; (2) sulfhydryl compounds, which utilize intra-and extra-cellular oxygen; (3) cysteamine, which acts by stimulating recovery mechanisms. The last-named substance also complexes with nucleic acid, and may thus preserve its integrity in the cell by preventing the formation of cross linkages (Hollaender and Stapleton, 1956).

Summarizing, studies with E. coli suggest that chemical radiation protection acts either by protecting the microorganism from radiation or by stimulating the recovery by repairing the lethal damage.

Cellular Regulation

The first actual evidence for the hypothesis that genes regulate metabolism by controlling the structure of enzymes came from the work of Beadle and Tatum (1951) with biochemical mutants of Neurospora. Ames and Garry (1959) discovered that the presence of histidine in Salmonella cells repressed the production of the enzymes needed for its biosynthesis.

Jacob and Monod (1961a,b) visualize the regulator system of the cell as being composed of three distinct genetic units. The first is the unit of structural genes which are responsible for the transcription of the genetic message, through RNA, to the protein molecule. The second unit is the operator gene, which together with the structural genes form the "operon". The operator gene gives the signal for the structural genes to function. The third unit is the regulator gene. This unit produces cytoplasmic material which acts in one of two ways. If the cytoplasmic material is a repressor substance it reacts with the operator to prevent the structural genes from functioning. In the "inducible enzyme system", the inducer reacts with the repressor material thereby mitigating the inhibition of the operator, by the repressor material (i.e. the structural gene is able to function).

In the second system (the repressible enzyme system) the regulator gene constantly produces material which has no effect on the operator gene. However, if this material reacts with repressor substance it becomes activated,

so that it reacts with the operator gene, inhibiting its action. The result is the cessation of the function of the structural genes. At the same time Jacob and Monod suggested that the regulator gene material was probably RNA, but in a later publication (Monod, Changeux, and Jacob, 1963) they implicated proteins are being more likely. This has been confirmed by the recent isolation of the lac repressor by Gilbert and Miller-Hill (1966) and the lambda repressor by Ptashne (1967).

If a mutation were to occur so that the repressor was prevented from reacting with the operator, the effect would be that the enzymes (either inducible or repressible) would appear to be constitutive. Such a mutation could take place in the regulator gene (which produces the material) or in the operator gene which receives the material (Jacob and Monod 1961 a, b).

Mutations of a structural gene that abolish or alter enzyme function result in either the production of no protein or of structurally modified protein. In such a case the biological pathway would no longer be operative unless the enzyme were compensated for by other means (see Whitehouse, 1965).

Other evidence (McFall and Mandelstam, 1963; and Harris and Sabath, 1964) suggests the possibility that the regulation of some enzyme synthesis occurs at the level of translation of the messenger RNA (m-RNA) into polypeptides. Umbarger (1962) suggests inhibition of the initial step (s) in a biosynthetic

pathway by the end product of the reaction chain, as a third possible mechanism of regulation. Monod and Jacob (1962) term this "allosteric inhibition", as the inhibitor is not a steric analogue of the substrate.

In summary the control of enzyme activity may occur at the following levels of cellular organization: (from Whitehouse , 1965)

- 1) Control of enzyme synthesis at transcription, that is, m-RNA formation from DNA ("Operon system", Jacob and Monod, loc. cit.).
- 2) Control of enzyme synthesis at translation, that is polypeptide formation from m-RNA (the penicillinase system in B. cereus, Harris and Sabath, loc. cit.).
- 3) Direct control of enzyme activity after synthesis ("end product inhibition", Unbarger, loc. cit.).

MATERIALS AND METHODS

Organism

The organism used throughout these experiments was Micrococcus violagabriellae isolated originally by Castellani (1955). In addition, Payne and Campbell (1965b) obtained two mutants of this Parental (Pig) strain. These authors designated the mutants as "Superpig", and "Littlepig"* on the basis of the color of the colonies produced on plates or slants containing an excess of iron. (Figure 6).

Incubation

For viability counts, plates were incubated at 30° C for 48 hours before being counted on a New Brunswick Electronic Colony Counter.

Liquid cultures were incubated in either a shaker or a shaker water bath which was kept at a temperature of 30° C.

Maintenance of Stock Cultures

Stock cultures were maintained on Basal Medium at 4° C. Transfers were made every 2 or 3 weeks.

* The three strains were kindly supplied by Dr. J. N. Campbell of the Department of Microbiology, University of Alberta.

Figure 6 The three strains of M. violagabriellae.
(a) Superpig, (b) Pig, (c) Littlepig.



Media

Basal Medium Plates (Payne and Campbell, 1965a)

1% Casamino acids (Difco)

0.03% Yeast extract (Difco)

0.01% Cysteine

0.01% Tryptophan

0.01% Asparagine

0.5% Glucose

0.5% Sodium Citrate

0.5% KH_2PO_4

0.5% NaCl

0.014% $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$

0.08% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$

1.5% Agar (Difco)

0.01% FeCl_3 (when present)

For liquid cultures, 0.5% sucrose was substituted for glucose.

MacDonald (1966) found that the Parental strain produced as much pigment in shake cultures as on agar slants if sucrose replaced glucose. In the present study this was also found to be true for the Superpig strain.

In the isotope experiments radioactive iron $^{59}\text{Fe}^*$ was added as a

* ^{59}Fe was supplied by Mrs. D. L. Weijer of the Department of Radiology, University of Alberta Hospital, Edmonton.

supplement to the iron already present in a concentration of $0.15 \mu\text{c/ml}$ of medium.

Buffer

A Sørensen's M/15 phosphate buffer (pH 7.2) was prepared according to Stittclough and Branham (1948). It contained 720.0 ml of a 0.947% anhydrous Na_2HPO_4 solution and 280.0 ml of a 0.908% KH_2PO_4 solution per litre.

Scintillation Solution (Zeigler, Chleck and Brinkerhoff, 1958).

15 ml 98.6% Ethanol

3 ml toluene containing 0.3% PPO (2,5-diphenyl-oxazole) and 0.01% dimethyl POPOP (4-methyl -5 phenyloxozlyl-benzene) both supplied by Packard Co.

Cells were suspended in the above solution to which subsequently 0.5 grams of Thixotropic Gel Powder (Packard) was added. Polyethylene counting vials (Packard) were used.

Irradiation

All irradiations were carried out using the Gammacell of the Radiation Laboratory of the University of Alberta. The average dose rate* used was approximately 11,200 rad/min (the rad is defined as the unit of absorbed dose per ml of irradiated solution).

* The doseimetry was determined by Dr. Maurice Robinson of the Dept. of Chemistry, University of Alberta, using the Fricke ferrous sulfate method (see Allen, 1961).

In addition to the dose received, 400 rads (according to the timing mechanism) was accumulated by the sample each time it was moved into or out of the field of radiation.

Survival Determination

Experimental cultures were prepared by spreading 0.1 ml of a saturated overnight broth culture onto twelve agar plates, six of which contained basal medium, and six of which contained basal medium supplemented with FeCl_3 . These plates were incubated for 18 hours. The cells were then washed from five plates of each type using 3 ml of sterile phosphate buffer at pH 7.2 per plate. The suspensions were centrifuged for 15 min at 1000 rpm to sediment clumped cocci. The supernatants were decanted and adjusted with sterile phosphate buffer (pH 7.2) to an optical density (O.D.) of 1.0 on a spectrophotometer (wavelength = 650 m μ). The O.D. value of 1.0 was equivalent to about 1×10^9 cocci/ml. Fifteen ml of each suspension were cooled in ice for 5 minutes and then irradiated for timed intervals up to a maximum of 9 minutes (maximum dose = 100,000 rads). Ten-fold dilutions of the irradiated samples were made in buffer, and 0.1 ml samples of the appropriate dilutions were spread on plates. Platings were carried out in triplicate for each of the two media, and the colonies on the plates were counted after 48 hours of incubation at 30°C. The percentage of surviving cells was calculated in the following manner:

$$\begin{array}{c} \% \\ \text{surviving} \\ \text{cells} \end{array} = \frac{\text{Avg. no of colonies on 3 plates} \times \frac{1}{\text{dilution}}}{\text{Avg. no of colonies on 3 unirrad-} \times \frac{1}{\text{dilution}}} \times 100$$

The percent of surviving cells (on a logarithmic scale) versus the dose of γ -rays (in drad) administered to the sample (on a linear scale) was plotted. The above procedure was repeated at least three times for all three genetic strains.

Radioactive Iron Uptake Study

Saturated overnight broth cultures of the Superpig, Parental and Littlepig were grown from agar slants. A 0.3 ml sample of each of the three strains was inoculated into flasks containing 30 ml of broth and $0.15 \mu\text{C/ml}$ of ^{59}Fe in the form of Fe^*Cl_3 . Non-radioactive FeCl_3 was added as a carrier, so that the final concentration was 0.01% ferric ions. A control, containing 0.01% ferric ions, was also set up using 0.3 ml of an overnight broth culture of the Parental strain; this latter culture was used to determine the incidence of background counts.

At predetermined intervals (0, 8, 10, 12, 14, 16, 18, 20 and 48 hours) a 2.5 ml sample was removed from each culture and added to 2.5 ml of buffer. The cells were then washed and centrifuged for 10 minutes at 10,000 rpm (12,100g) in a refrigerated centrifuge. Two ml of the supernatant were removed and added to 18 ml of scintillation solution in a counting vial. The remainder of the supernatant was decanted and the cells re-suspended in 10.0 ml of buffer.

The cells were then washed and 1.0 ml was removed and added to 4.0 ml of buffer. The O.D. of this solution was determined in a spectrophotometer (wavelength = 650 m μ).

The O.D. readings and the counts per minute (see below) versus time of incubation in the presence of radio-active FeCl₃, were plotted on semi-logarithmic paper (see Figure 9). In addition, the O.D. readings of a Parental strain culture (grown in the presence of non-radioactive FeCl₃ only) were used to determine the effect of radioactive FeCl₃ on the growth of the organism. No significant differences in growth performance were found to occur.

Since no effort was made to remove cell clumps of M. violagabriellae when in suspension, the O.D. was not used as a quantitative measure of growth of the organism, but rather as an indication as to whether or not normal growth was taking place in the presence of radioactive iron.

The remaining 9.0 ml were re-centrifuged (10,000 rpm for 10 minutes) and 2.0 ml of the supernatant were removed and placed in a counting vial containing 18 ml of scintillation solution. The remainder of the supernatant was decanted and 10.0 ml of buffer was used to re-suspend and to wash the cells. After centrifugation (10,000 rpm for 10 min), 2.0 ml of the supernatant were removed and placed in a counting vial containing 18 ml of scintillation solution. The remainder of the final supernatant was then decanted and the cells were re-suspended in 2.5 ml of buffer. A 2.0 ml sample was removed

and placed in counting vials containing 18 ml of scintillation solution and 0.5g of thixotropic gel powder.

A diagrammatic representation of this procedure is given in Figure 7.

The counting was carried out using a Packard Liquid Scintillation Counter with a counting efficiency of 7.1 percent. For the determination of the amount of quenching by the cells, a ^{232}Ra automatic external standard was counted after each sample. (Schrodt, Gibbs, and Cavannaugh, 1965).

Figure 7. Diagrammatic representation of the "Radioactive Iron Uptake Study" procedure.



- solution transfer



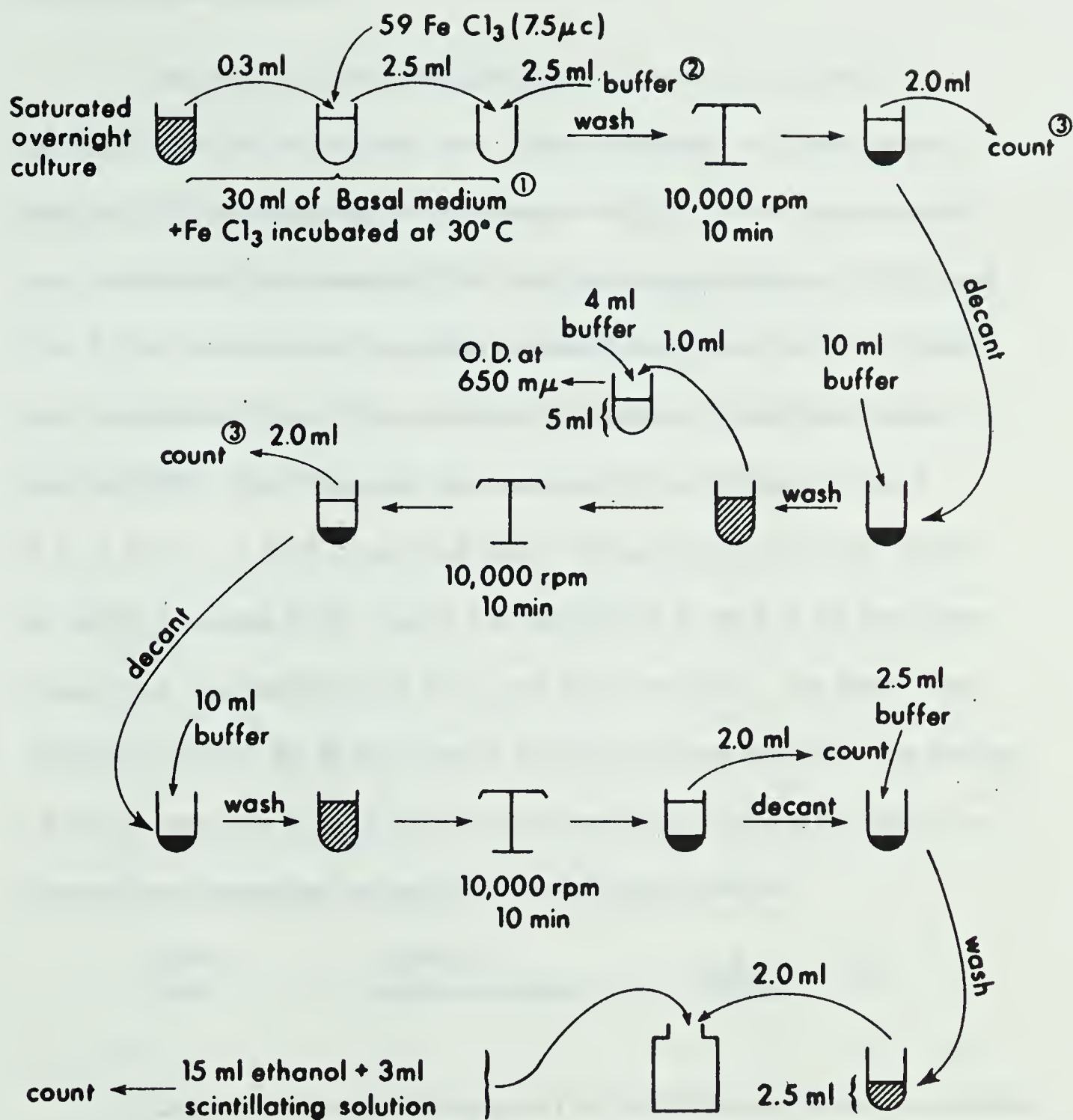
- flask



- counting vial



- centrifugation



1. Basal medium contains sucrose in place of dextrose.
2. Sorenson's M/15 phosphate buffer ($\text{pH} = 7.2$).
3. 2.0 ml of supernatant was placed in a counting vial containing 15 ml of ethanol and 3 ml of scintillating solution.



Diagram 12: A flowchart showing the process of...
 (1) ... (2) ... (3) ...
 ...
 ...

Post-Irradiation Growth

One-half ml of an overnight broth culture was inoculated into 50 ml of broth with and without iron. After incubation in a shaker water-bath at 30°C for 6 hours (or 16 hours respectively)*, 15.0 ml were removed and irradiated in the Gammacell for 1 min (dose: approximately 12,000 rads). Two 0.5 ml samples were immediately removed and placed in 50 ml of broth with and without iron. These were again incubated in the shaker water-bath at 30°C. One ml samples were removed at the following times: 0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 hours. These samples were then diluted in sterile phosphate buffer, and 0.1 ml was spread on each of six agar plates (three were supplemented with FeCl_3 and three were not). The plates were incubated at 30°C for 48 hours before the colonies were counted. The number of viable organisms in the post-irradiation medium at the time the aliquot was removed was determined according to the following formula:

$$\text{viable cells} = \frac{\text{number of colonies counted}}{\text{dilution}} \times 10$$

Controls were made at the same time by following the above procedure and calculations, but using equal volumes of unirradiated cells taken from the same cultures as the test samples. All experiments were repeated at least 3 times.

* The uptake of large quantities of FeCl_3 by Parental and Superpig strain cells (prior to irradiation) can be delayed by using 6 hour cultures.

RESULTS AND DISCUSSION

A. Parental Strain

A relatively low resistance to ionizing radiation ($D_{37} = 13$ krad) was found when the Parental strain of M. violagabriellae was grown on a pre-irradiation medium which was not supplemented with iron. If the post-irradiation medium contained iron (0.01% Fe Cl_3) the resistance was found to be slightly greater ($D_{37} = 17$ krad, see Figure 8).

The presence of iron in the pre-irradiation medium (irrespective of the iron content of the post-irradiation medium) enhanced radio-resistance ($D_{37} = 28$ krad) in such a manner that the resulting sigmoid survival curve contains a shoulder indicating resistance to low doses of irradiation. No radiation effect on the viability of the cells was observed for doses up to 24 krad. However, on increasing the dose, the survival curve became exponential and parallel to the slope of the survival curve of cells grown in a pre-irradiation medium without added iron.

Associated with the resistant shoulder is a radiation effect on cell viability. It was found that doses between 12 and 24 krad increased the percentage of surviving cells to 108%. That is to say, the viability count of irradiated cells was 8% higher than the viability count of a non-irradiated sample from the same culture. Several explanations can be

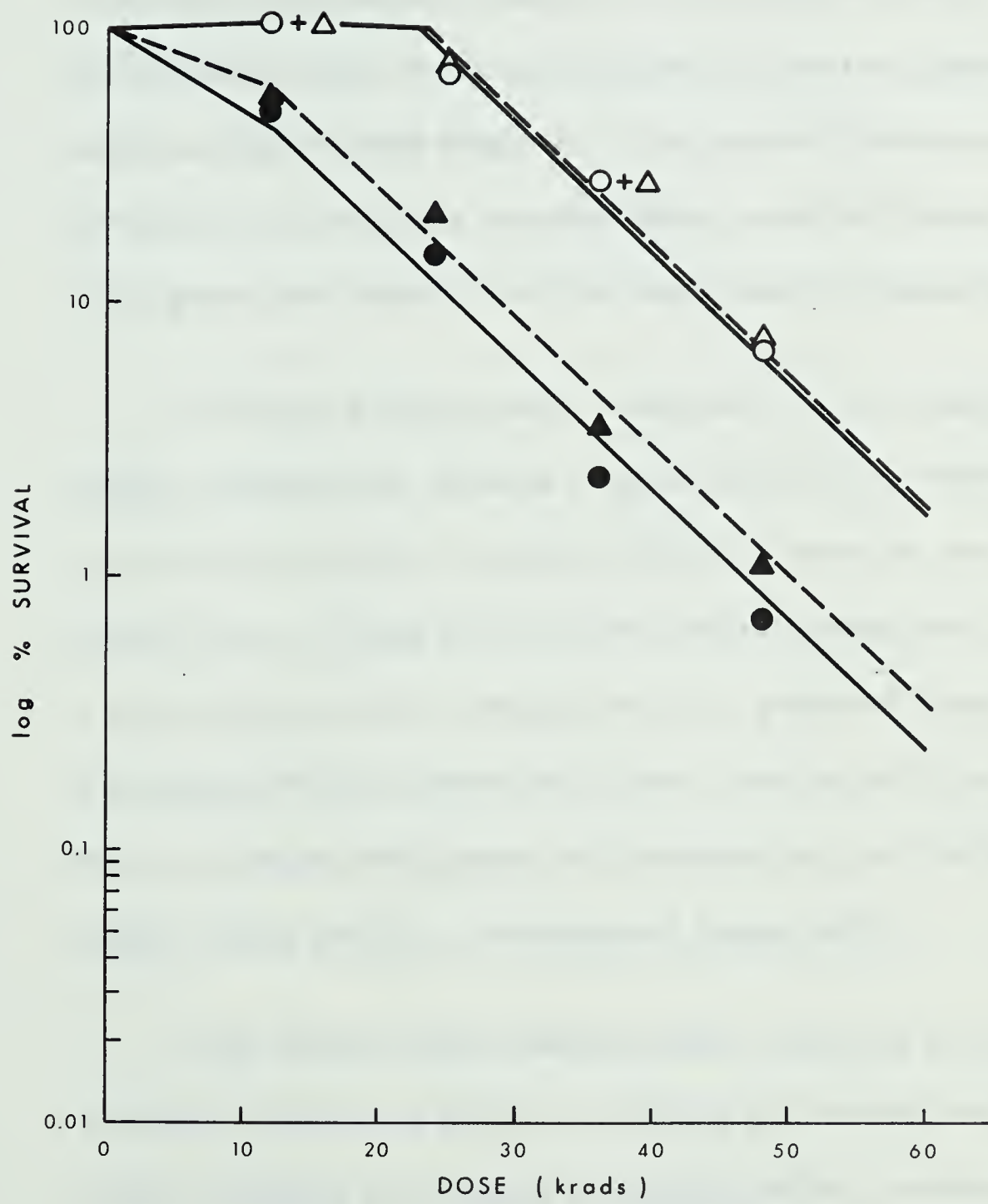
advanced:

- (i) Cells in suspension were found to clump when observed by means of phase contrast microscopy. Although centrifugation was used to remove clumped cocci from the suspension, paired cells may not have been affected by the centrifugal force applied and hence remained a part of the population of cells to be irradiated. On the assumption that radiation enhanced separation of the cell pairs, an increase in the viability count can be expected.
- (ii) It can be expected that a sample of cocci represent a population of cells in different stages of development with some in the process of division. Upon radiation, a dose of 12 krad stimulates the cells into completing their division, yielding two daughter cells. It is not believed that cells in the early stages of division are removed from the suspension by centrifugation: their density is not significantly greater than that of non-divided cells.
- (iii) Weijer (1963) found that certain doses of X-rays stimulated the germination of Neurospora crassa conidia. Reproductive stimulation of bacterial cells in a state of physiological death by low doses of γ -rays would account for the increase in viable counts obtained.

Figure 8. The effect of γ -ray irradiation on Parental (Pig) strain M. violagabriellae cells.

	Pre-irradiation medium	Post-irradiation medium
$\Delta - - - - \Delta$	Basal + Fe	Basal + Fe
$\Delta - - - - \circ$	Basal + Fe	Basal
$\blacktriangle - - - - \blacktriangle$	Basal	Basal + Fe
$\bullet - - - - \bullet$	Basal	Basal

Pig



B. Superpig Strain

Upon radiation, Superpig cells grown on media not containing iron, produced results similar to those obtained for the Parental strain. A relatively low resistance to radiation was noted ($D_{37}=13$ krad). A post-irradiation supply of iron in the medium gave rise to a higher radio-resistance ($D_{37}=18$ krad) (Figure 9). The exponentially decreasing slopes of the survival curves of the irradiated Parental strain and Superpig strain (when grown under identical conditions) were found to be almost identical.





Irradiation of Superpig cells, when grown in a pre-irradiation medium containing iron, produced a sigmoid survival curve characterized by an extensive shoulder of resistance (28 krad). As was the case for the Parental strain, the slope of the survival curve for Superpig cells, raised on a pre-irradiation medium containing iron (i.e., pigmented Superpig cells) and radiated with doses greater than 28 krad, coincided with the slope of the curve obtained with Superpig cells maintained on a pre-irradiation medium lacking iron (i.e., non-pigmented Superpig cells).

Cells raised on a pre-irradiation medium containing iron and after irradiation maintained in a medium containing iron, showed an increase of 20% in viability over Superpig cells treated similarly but maintained on a post-irradiation medium lacking iron (Figure 9). This increase in viability was accounted for in the same manner as outlined on page 37 for cells of

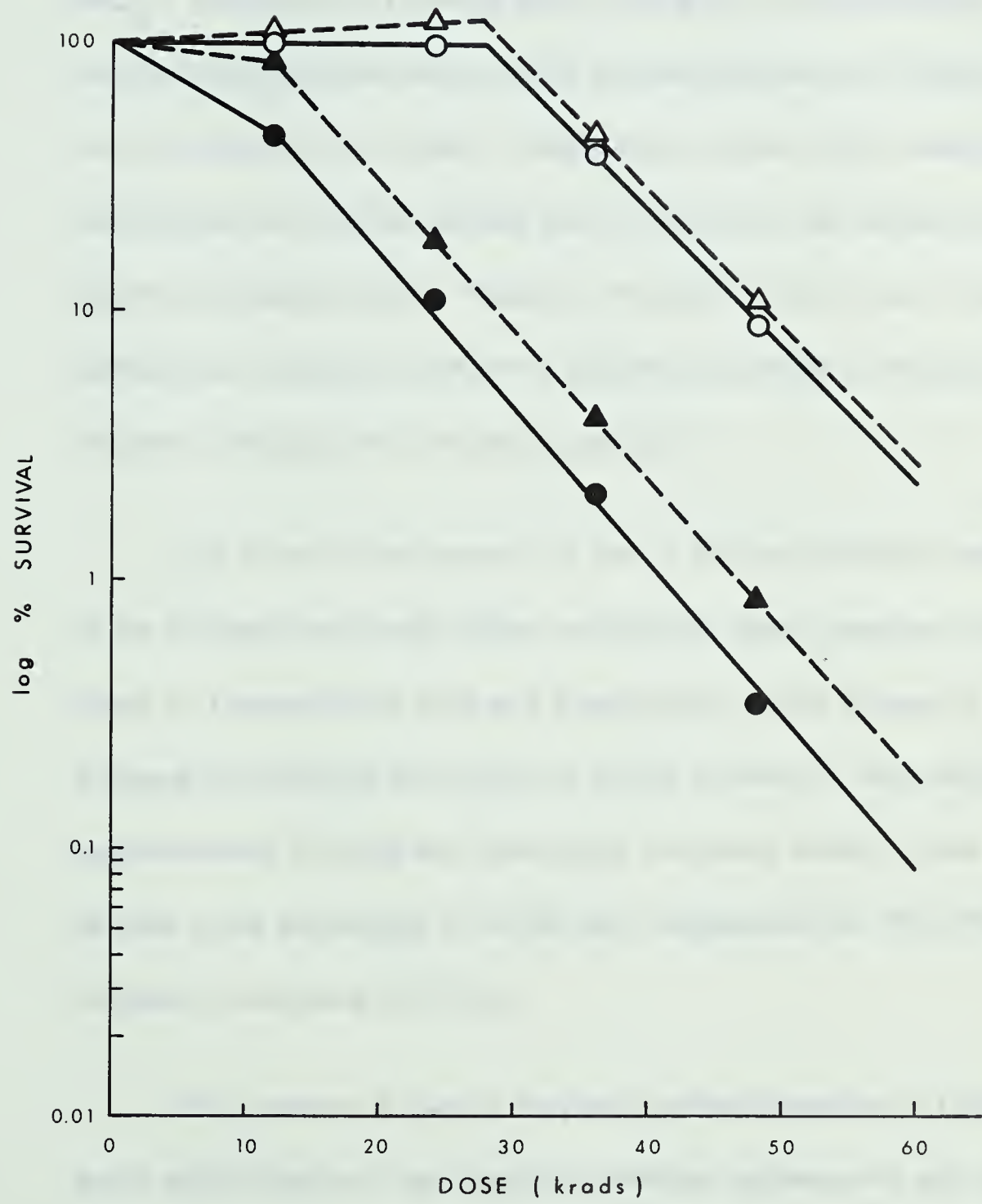
the Parental strain. No increase in viability count was detected (99% at 12 krad and 98% at 24 krad) when FeCl_3 was omitted from the post-irradiation medium.

When compared with Parental strain cells, Superpig strain cells contain a greater amount of violet-red pigment (Payne and Campbell, 1965b). Consequently, differences in pigment concentration may underlie the post-irradiation effect on cell viability. It is believed that Parental strain cells remove more iron from the medium than is needed for pigment production, whereas Superpig strain cells utilize all, or nearly all, the accumulated iron for pigment production. A young Parental strain cell raised on a medium containing iron but transferred into a new environment now lacking iron, is able to utilize the excess iron (which it brought with it from the original environment), until it is able to adapt to the new conditions imposed upon it. Superpig strain cells however, do not contain free (excess) iron and hence experience difficulties in their adaptation to the new (iron-free) environment. Hence, the presence of iron in the post-irradiation medium enhances the ability of Superpig strain cells to recover from the lethal effects of ionizing radiation, regardless of whether or not iron was present in the pre-irradiation medium. The presence of iron in the pre-irradiation medium of the Superpig organism confers upon the cell a high degree of radio-resistance.

Figure 9. The effect of γ ray irradiation on Superpig strain M. violagabriellae cells.

	Pre-irradiation medium	Post-irradiation medium
	Basal + Fe	Basal + Fe
	Basal + Fe	Basal
	Basal	Basal + Fe
	Basal	Basal

Super pig



C. Littlepig Strain

The Littlepig strain is characterized by its inability to produce violet-red pigment, when grown on a medium supplemented with 0.01% FeCl_3 . Irradiation of Littlepig strain cells grown on basal medium (i.e. lacking iron) produced results similar to those obtained with Superpig strain cells and Parental strain cells. That is to say, when after irradiation cells were plated on a medium lacking iron, a relatively low resistance to γ -rays ($D_{37} = 13.5$ krad) resulted. However, the availability of iron in the medium post irradiation produced a significant increase in the percentage of viable cells ($D_{37} = 17.5$ krad) (Figure 10).

The effect of the presence of iron in the pre-irradiation medium of the Littlepig strain cells differs considerably when compared with its effect on Parental strain cells and Superpig strain cells (Figure 10). Although for Littlepig strain cells an initial increase in radio-resistance (approximately 20 krad) was observed, on increasing radiation dose a rapid decline in the percentage of viable cells compensated for this initial increase in resistance to γ rays.

The presence of iron in the post-irradiation medium of Littlepig strain cells raised on a pre-irradiation medium supplemented with iron, increased the percentage of cells capable of producing visible colonies. The difference in radio-sensitivity between Littlepig strain cells incubated post irradiation on an iron supplemented medium ($D_{37} = 27$ krad),

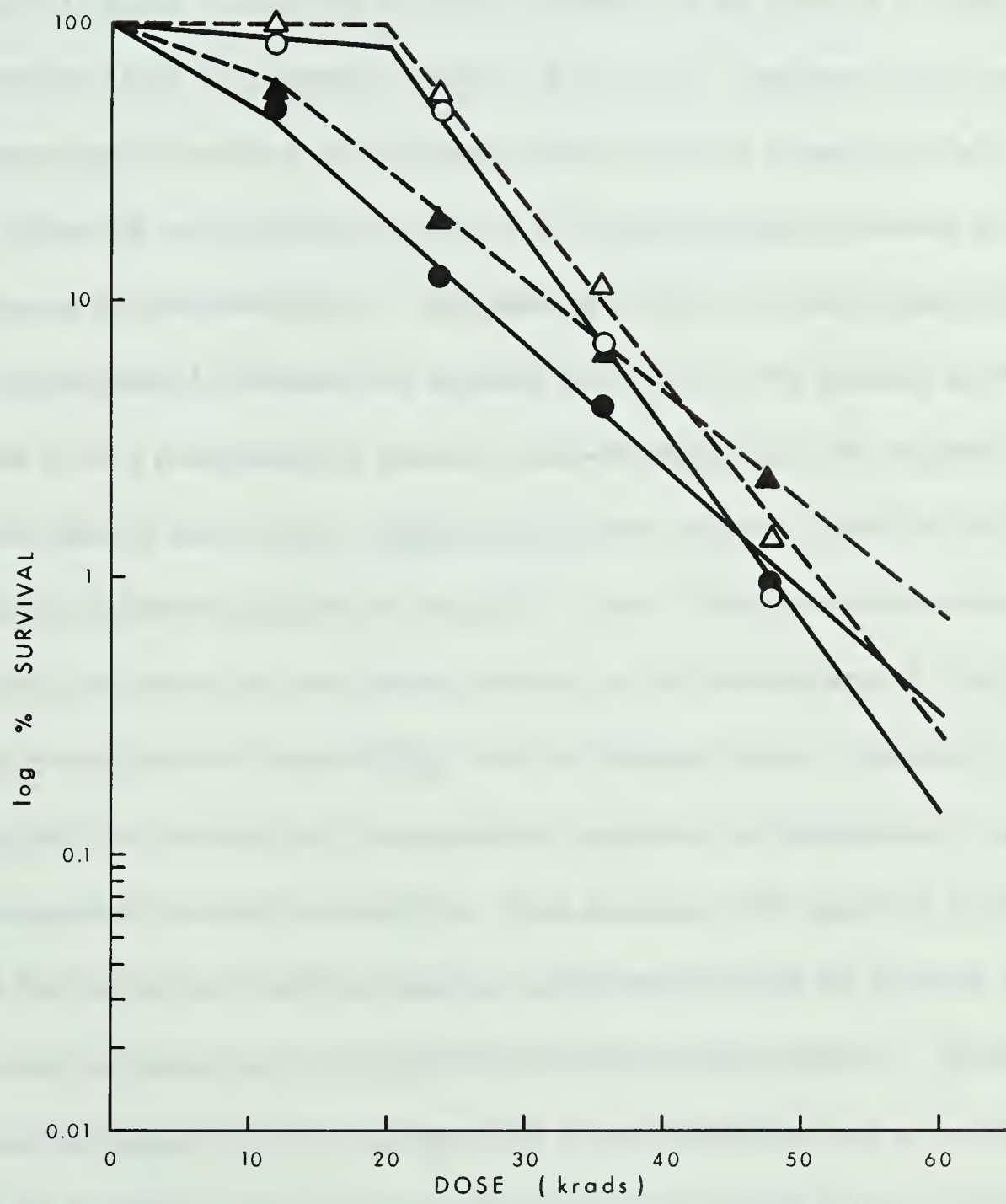
and Littlepig strain cells maintained on a basal post-irradiation medium ($D_{37} = 25$ krad), is not significant.

According to Campbell (1966) the Littlepig organism differs from the Parental strain cell only in the inability to produce the complete pigment when grown on medium containing iron. Consequently, Littlepig cells when grown in the presence of iron, probably contain a precursor of the violet-red pigment. From the data presented, it becomes evident that the complete violet-red pigment is not required for radiation resistance. The very fact that the presence of iron in the post-irradiation medium results in an increased survival is indicative of either an iron-induced, or iron-stimulated pigment/pigment precursor production.

Figure 10. The effect of γ ray irradiation on Littlepig strain M. violagabriellae cells.

	Pre-irradiation medium	Post-irradiation medium
$\Delta - - - - \Delta$	Basal + Fe	Basal + Fe
$O - - - - O$	Basal + Fe	Basal
$\blacktriangle - - - - \blacktriangle$	Basal	Basal + Fe
$\bullet - - - - \bullet$	Basal	Basal

Littlepig



Mechanisms of Protection

In discussing the possible protective mechanisms underlying the radio-resistance of M. violagabriellae when exposed to γ -irradiation, it has to be borne in mind that the radio-resistance of the organism is directly correlated with the exogenous supply of ferric ions. Experiments using cell suspensions of the three strains clearly indicate that the presence of FeCl_3 in either the pre-irradiation medium or in the post-irradiation medium will increase the radio-resistance. The presence of the red-violet pigment which is accumulated by Parental and Superpig strain cells in the presence of Fe^{+++} ions is not a prerequisite for imposing radio-resistance upon the organism. All three genetic strains of M. violagabriellae show an active uptake of iron from the environmental medium (see page 53). In two of the three strains the accumulated iron enters the biochemical pathway for pulcherriminic acid, the pigment associated with the Parental (Pig) - and the Superpig strain. However in Little-pig cells the pulcherriminic acid pathway appears to be blocked and a colourless pigment precursor accumulates. Data presented with respect to the effect of iron in the pre-irradiation medium, would indicate that the presence of pigment (as well as pigment precursor substance) enhances radio-resistance. However, the results of experiments relating the effect of post-irradiation iron on radio-resistance of the organism, seem to indicate that the protecting agent is the tri-valent iron ion. In other words, the violet-red pigment can only be regarded as a radiation protector, because of the fact that this substance contains Fe^{+++} as a central atom.

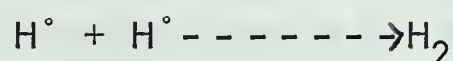
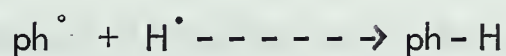
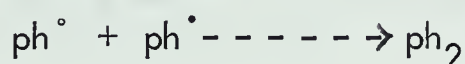
Immunological studies by Campbell et al. (1964) suggest that the pigment pulcherriminic acid is associated with the cell-wall or membrane fraction of the cell. Consequently, the main protective mechanism of M. violagabriellae (as it is represented by the iron containing pigment) appears to be localized in this sub-cellular structure. Although it is unlikely that the remainder of the cell lumen is devoid of iron, the above representation may serve as a model.

The association of iron-containing pigment with the cell-wall or membrane appears to be indicative of the existence of a radiation barrier. Although a radiation barrier can be visualized, one has to remember that such a barrier is of no consequence with respect to the primary action of ionizing radiation, which in the case of ^{60}Co has an energy in excess of 1 MeV. However, such a radiation barrier may influence the secondary effects of ionizing radiation, by acting as a 'scavenger' for free radicals produced in the lumen or immediately outside the cell. The precursor substance is produced when Littlepig cells are grown on media supplemented with FeCl_3 . The cells may therefore contain ferric ions in their lumen, and hence, the radiation protection qualities of cells possessing the pigment precursor substance is in fact due to the presence of ferric ions in the cell lumen. Studies by Schwarz and Hritz (1958) indicate, that the presence of ferric ions in an aqueous solution can reduce the number of free radicals produced. Collinson, Dainton and McNaughton (1957) found that ferric ions act as efficient linear terminating agents. That is to say, if

ferric ions are present when organic radicals are irradiated, the ferric ions take up the free electrons, leaving terminated chains and ferrous ions.

Baxendael and Smithies (1955), using a system containing organic compounds, report a significant ferric ion reduction upon radiolysis. The latter authors suggest the following reactions for an aqueous system containing benzene

(ph=phenol ring):



The reaction in the presence of ferric ions:

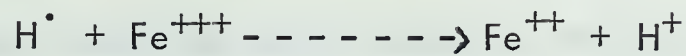


Spinks and Woods (1964) on the other hand believe that Fe^{+++} react directly (as 'scavengers') with hydrogen free radicals yielding hydrogen atoms:



On continuous irradiation, the ferric ions compete with the ferrous ions

for hydrogen and hydroperoxyl radicals in the following manner:



Eventually a steady state concentration of ferric ions is reached where the rates of oxidation and reduction are equal.

The presence of chlorine free radicals will cause ferrous ions to react in the following manner (Spinks and Wood, 1964).



The presence of chloride ions in the cell would have no appreciable effect on the cells' ability to survive the indirect effects of irradiation, as chloride ions are only effective in reducing and reacting with free radicals at high acid concentration (Sworski, 1955).

The effect of FeCl_3 in the pre-irradiation medium on the radio-resistance of the three strains employed can be summarized as follows:

Table I The effect of the composition of the pre-irradiation medium on the radio-resistance of three genetic strains of M. violagabriellae (basal post-irradiation medium).

Strain	D_{37} for basal medium	D_{37} for basal medium supplemented with FeCl_3
Parental (Pig)	13 krad	24 krad
Superpig	13 krad	28 krad
Littlepig	13.5 krad	20 krad

As can be seen from Table I the protective effect of FeCl_3 as an additive to the pre-irradiation medium causes an increase of approximately 100% in the D_{37} for all strains (the actual increase amounts to 11, 15 and 6.5 krad respectively). The magnitude of these increments in radio-resistance are such, that it is highly unlikely that the 'scavenger' effect of ferric ions solely underlies the protective mechanism. In other words, it is unrealistic to assume that the concentration of ferric ions within the cell-lumen (of which a considerable amount is concentrated in outer cell structures) is sufficient to account for such a highly effective protection mechanism.

The observation, that the presence of iron in the post-irradiation medium will confer slight resistance upon the cell can be regarded as additional evidence that more than one protective mechanism is involved.

Table II The effect of FeCl_3 in basal post-irradiation medium on the radio-resistance of three genetic strains of M. violagabriellae (basal pre-irradiation medium).

Strain	D_{37} for basal post-irradiation medium	D_{37} for basal post-irradiation medium supplemented with FeCl_3
Parental (Pig)	13 krad	17 krad
Superpig	13 krad	18 krad
Littlepig	13.5 krad	17.5 krad

Table II shows the influence of post-irradiation iron on the D_{37} of

the three different genetic strains. The availability of Fe^{+++} ions in the post-irradiation medium gives rise to an increase of the D_{37} indicating, that the Fe^{+++} ion is important for the repair of the radiation damage.

Because of the fact that this iron is supplied to the cell as a post-radiation treatment, at a time that there are no free radicals present, the protective action of iron is no longer that of a free radical scavenger.

Recent studies dealing with the biological effects of ionizing radiation have emphasized its influence on the cytoplasmic membrane. Hutchinson (1960) in his literature review concluded that ionizing radiation may well enhance the process of active transport through a cell membrane at much lower doses than are required to facilitate diffusion (Danielli, 1958). O'Brien (1960) showed that the efflux of potassium and of phosphate from X-irradiated yeast was affected differently by the addition of glucose. However, whether glucose was present or not, leakage rates of phosphate and potassium ions from irradiated cells were higher than from the unirradiated controls. Since metabolic activity is not essential for this leakage, the loss of cellular constituents may be regarded as primarily a diffusion phenomenon rather than an active transport process. His data indicated that radiation increases the number of sites through which materials diffuse out of the cell. Sulfhydryl groups in the membrane were shown to be radiation-sensitive. The ability of bivalent and multivalent anions and cations to decrease cellular permeability has been discussed by De Haan (1935) and Höber (1946). These ions have been found to be effective radio-protectors (Weijer, 1962).

Bungenberg De Jong and Bonner (1935) described phosphatide autocomplex coacervates as ionic systems with the structure and function of the protoplasmic membrane. The effect of ionizing radiation on these structures manifests itself in 'cross-linkage' of the constituent molecules. Cross-linkage of membrane molecules will undoubtedly disrupt the osmo-regulatory structure at the point of impact, which in turn will give rise to an additional site through which diffusion of cell constituents may occur. Repair of these membrane perforations by electric charge attraction will mitigate the leakage of intracellular substances and consequently increases the chance of survival.

The presence of Fe^{+++} ions in the outer membrane structures facilitates repair, since it is assumed that radiation damage to the osmo-regulatory structures concerns either the pigment bearing membrane (i.e., Fe^{+++} containing) or a cellular membrane structure adjacent to it. The availability of Fe^{+++} ions close to the site where repair has to be initiated is believed to be of overriding importance with regard to the effectiveness of such a repair. But even exogenous iron present in the post-irradiation medium is of importance. The presence of this source of Fe^{+++} ions increases the D_{37} with approximately 3 krad. It is interesting to note that the effectiveness of post-irradiation iron is independent of the genetical strain employed and hence independent of the cellular uptake and the quantitative aspects of pigment or pigment precursor production: a phenomenon which on the basis of the above hypothesis can be expected to occur.

In summary, it is suggested that the radioprotective action of FeCl_3 on cells of M. violagabriellae is brought about by at least two different mechanisms:

- (i) Ferric ions act as free radical scavengers. The final yield of this process depends on the availability of intra-cellular iron and hence is only partly dependent on the quantitatively genetic trait for pigment/pigment precursor formation.
- (ii) Ferric ions by means of charge neutralization prevent excessive loss of cell constituents caused by the radiation-induced increase in cell permeability and therefore lessen the death rate of γ -irradiated bacteria. This process is dependent upon the intra-cellular level of ferric ions as well as upon the presence of exogenous iron.

Radioactive Iron Uptake Studies

The amount of labelled iron taken up by the cells correlated positively (as determined visually) with the amount of violet-red pigment produced. For example, the saturated culture of the Superpig strain which produced the largest amount of visible pigment, took up the equivalent of 5.4×10^4 counts/min of radioactive iron, while the saturated culture of the Parental strain which produced a lesser amount of visible violet-red pigment took up the equivalent of 2.1×10^4 counts/min, and the saturated culture of the Littlepig strain which produced no visible violet-red pigment took up the equivalent of 6.4×10^2 counts/min. Evidence has been found to support that the saturated cultures of the three strains contain equal numbers of cells and there is no reason to suspect that there is selective uptake of the labelled iron by any of the three genetic strains employed (Table III and Figure II).

The graphs (Figure II) indicate that the O.D. increased slowly during the period when the three strains employed exhibited maximum iron uptake (8 - 12 hr. for the Parental-strain, 6 - 10 hr. for the Superpig-strain, and 8 - 12 hr. for the Littlepig-strain). During the period of rapid uptake of iron (for the Parental strain) or immediately after this period (for the Superpig-and the Littlepig-strains), large increases in the O.D. readings were observed due to the formation of the optically dense pigment.

Table III

Calculation of the actual counts per minute of radioactive iron
(^{59}Fe) taken up by the three strains of M. violagabriellae.

TABLE III

background = 116 c/m

LITTLEPIG

SUPERPIG

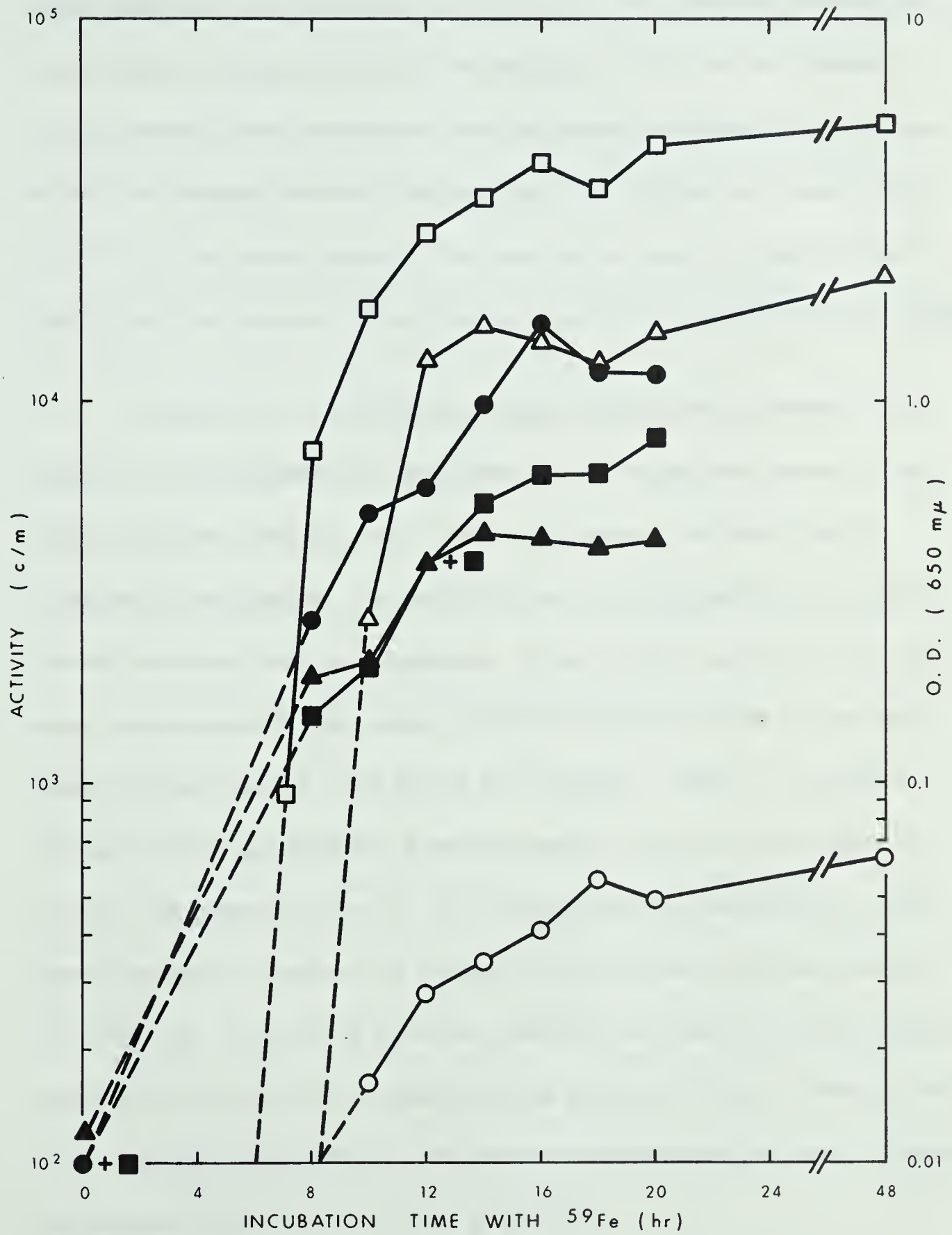
PARENTAL

Time of Incubation in Presence of Fe	Recorded Counts Per Min	100 - % Quenching	Actual c/m -background	Recorded Counts Per Min	100 - % Quenching	Actual c/m -background	Recorded Counts Per Min	100 - % Quenching	Actual c/m -background
0	98		0	77		0	138		22
7	108		0	940	90.0	940	175	95.0	62
8	113		0	6,535	87.1	7,370	188	95.0	76
10	2,596	90.9	2,728	13,999	78.9	17,596	249	95.0	140
12	10,618	81.8	12,839	19,616	70.6	27,762	385	94.1	286
14	12,502	79.1	15,659	22,384	65.6	33,791	432	93.3	339
16	11,829	82.3	14,232	25,653	60.3	42,350	495	93.7	404
18	10,062	81.0	12,279	23,171	63.5	36,307	648	94.0	566
20	12,091	79.6	15,044	26,503	56.5	46,703	568	91.8	492
48	15,544	72.2	21,368	26,491	48.8	54,252	706	92.3	639

Figure II

The uptake of iron and the optical density ($\lambda = 650 \text{ m}\mu$) of the three strains of M. violagabriellae incubated in the presence of radioactive iron (^{59}Fe).

- - - - - extrapolated results
- experimentally obtained results
-● O.D. of Littlepig
- ▲.....▲ O.D. of Parental
-■ O.D. of Superpig
-○ c/m of Littlepig
- △.....△ c/m of Parental
- ▢.....▢ c/m of Superpig

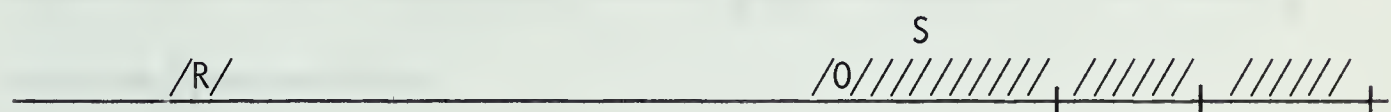


The evidence presented here suggests, that the growth of the organism either stops or at least slows down greatly while iron is removed from the media by the cells. A change in color of the media (to violet-red) was observed to have started in both the Parental and the Superpig cultures within two hours of the first detected uptake of labelled iron (i.e. 9 hr for the Superpig strain by 12 hr for the Parental-strain). This observation seems to suggest the possibility that the presence of iron induces the production of the violet-red pigment.

Campbell et al. (1964) have suggested that pigment synthesis is iron dependent, but independent of incorporation into the pigment molecule itself. The fact that the Littlepig strain is unable to produce complete pigment (Campbell, 1966) suggests the possibility that iron incorporation is essential for the violet-red color to be produced. If the synthetic pathway for the pigment involves several steps, some of which are associated with a cytochrome-linked electron transfer chain (Payne and Campbell, 1965a), it is possible that one of the steps (probably a terminal one) involves the incorporation of iron into the pigment molecule. The Littlepig cells may be defective in their genetic material to produce the enzyme for this function or for the production of a precursor. They would therefore, contain a precursor of the final pigment, and if the missing enzyme is supplied (in the presence of iron) to Littlepig cells or to an extract from Littlepig cells, pigment production might occur. Experiments are presently underway to study this possibility.

The manner in which iron is removed from the media and the concentration which it reaches in the cell, suggests the possibility that some sort of active transport is involved in the movement of iron ions across the membrane. A regulatory mechanism which is affected by the presence of iron is probably operative in the production of the pigment.

If an "Operon System" (Jacob and Monod, 1961a, b) were to be the control mechanism, it is assumed that such a mechanism is fully active in parental cells. That is to say, Parental cells possess a complete system including regulator, operator, and structural genes.



The "Operon System" of M. violagabriellae acts in one of two ways.

1. Induction

The regulator gene (R) constantly produces the apo repressor which normally inhibits the operator (O) from signaling the structural genes (S) to produce the enzymes which synthesize the pigment. The presence of iron in the cell prevents the regulator material from reacting with the operator gene. The now active operator signals the structural genes to function. The enzymes produced catalyze the biosynthesis of the pigment. One of the final enzymes may be responsible for the incorporation of iron into the pigment molecule (enzyme has been termed "Iron incorporatase" for lack of better terminology).

The removal of iron from the cytoplasm by the pigment allows all new regulator material to remain active to react with the operator and prevent further pigment production.

The Littlepig cells have an alteration in the genes controlling the enzyme(s) responsible for the active transport of iron ions into the cell, that is, iron can only be transferred in small amounts by simple diffusion. Consequently the amount of iron may not be sufficient to react with large amounts of repressor material. The end-result is the production of only incomplete pigment molecules: all the iron taken into the cell reacts with the repressor material and none is incorporated into the pigment molecules. The Littlepig cells are therefore white.

A second possibility is that the alteration concerns the structural gene controlling the production of one of the pigments biosynthetic enzyme. Large amounts of iron are not taken up by Littlepig cells because the iron transport enzymes are sensitive to the concentration of free iron ions in the cytoplasm (i.e. the failure of the utilization of Fe, due to the absence of the end product might serve to inhibit future uptake of Fe into the cell).

The Superpig cells may have an alteration in the regulator gene or the operator gene. The result is that the regulator material has no control over the operon, so the cell produces large amounts of incomplete pigment. When the cell stops dividing, it starts to actively remove iron from the medium and

move it into the cell where it is incorporated into the pigment molecules. The enzyme(s) responsible for the movement of iron into the cell are sensitive to the concentration of free iron ions present in the cytoplasm. If the iron is bound in the pigment molecule it is not free in the cytoplasm, therefore a large excess of iron ions will be taken up and moved into the cell.

2. Repression

In the Parental organism the regulator produces inactive repressor material. The structural genes produce constitutive enzymes which catalyze the production of the pigment. One of the last enzymes in the operon is "iron incorporatase" which is responsible for incorporating iron into the pigment molecule. The complete pigment reacts with the regulator material and as such inhibits the operator, which in turn prevents the formation of further enzymes. Consequently, pigment production ceases. Parental cells actively remove large amounts of iron from the medium at a very rapid rate (except during cell division), and immediately incorporate the iron into the pigment precursor. Large amounts of complete pigment result, which in turn react with the regulator material to inhibit the operator. Inhibition of the operator prevents the structural genes from producing further enzymes necessary for pigment biosynthesis.

In Littlepig cells an alteration in the genetic material which controls the transport of iron ions across the cell membrane and into the cell, has occurred. The lack of iron prevents the production of complete pigment, therefore only pigment precursor is produced.

The Superpig cells are altered in the regulator gene or the operator gene, which prevents the regulator material from reacting with the operator gene. The result is the lack of a shut off mechanism, and hence, the cell continues to produce pigment.

Other regulatory systems can be used to explain the synthesis or lack of synthesis of complete pigment in M. violagabriellae and amongst them the "end product inhibition" (Umbarger, 1962) and the "inhibition at translation" (Harris and Sabath, 1964) hypotheses remain attractive.

In the system of Umbarger, the genes controlling the production of the biosynthetic enzymes are constitutive. They produce a pigment precursor which reacts with iron (when it is actively taken up by the cell) to produce the final pigment which inhibits one of the early genes from producing its enzyme. Because linearity exists in the genes controlling the biosynthetic enzymes, all of these genes will be inhibited by the cessation of an initial (i.e., early) enzyme. The above system would apply to the Parental strain cells of M. violagabriellae.

In the Littlepig cells an alteration in the iron transport system prevents the movement of large amounts of iron into the cell, resulting in the lack of formation of complete pigment. The net result is, that large amounts of pigment precursor are produced but no final pigment. A second alteration in the gene which controls the iron incorporatase enzyme, may accentuate the lack of pigment. If the iron transport system is sensitive to the concentration of free iron in the cytoplasm then the lack of iron incorporation into pigment will enhance the sensitivity.

Superpig cells, on the other hand, contain an alteration in the initial gene resulting in insensitivity of the cell to the high concentration of the pigment (i.e., there is no shut off mechanism).

If inhibition at translation is the regulatory mechanism operative, the genes controlling enzyme biosynthesis are constitutive. In the Parental cells the final pigment inhibits the m-RNA from forming enzymes.

The Littlepig cells contain an alteration which concerns a gene controlling one of the enzymes involved in pigment synthesis. As a result of such a mutation, no substrate for subsequent enzymes will be formed.

The initial gene in the Superpig cells has been altered in such a way that it produces an enzyme which is still active in the biosynthetic pathway. The m-RNA however, is no longer susceptible to inhibition by the final pigment. The end-result is that greater amounts of pigment are produced when compared

with Parental strain cells.

Although the pattern of iron uptake by M. violagabriellae suggests that some sort of regulation mechanism is active, at the present time, no definite statement can be made about the mechanism of biological regulation with regard to the control of the synthesis or lack of synthesis of pulcherrimin pigment.

Post-irradiation Growth Patterns

This study was undertaken in order to determine the effect of the presence of iron (in the post-irradiation medium) on irradiated cells. Un-irradiated Parental strain cells (control) when grown in media for 6 hours, are in the logarithmic growth phase and hence in a dividing state. On transference to fresh medium of the same composition, no lag period can be detected (Figure 12) as they continue to divide at a rapid rate. Even transfer to medium differing in composition by a single factor does not affect the growth of the organism.

The 16 hour control cultures of the Parental strain of M. violagabriellae were characterized by the presence of a lag period after they were placed in fresh medium (Figure 13). It is assumed, that the lag in growth is indicative of the fact that the cells are in a resting state during that period. It is assumed further, that the length of the lag period corresponds with the adjustment time of the cells to the new medium. Termination of the lag period occurs after the period of adjustment at which time the cells start to divide.

Cells grown for 16 hours in medium lacking iron did not exhibit pigment and the lag period of growth was found to be short (approximately 30 min, Figure 13 a,b); however, when the initial medium contained FeCl_3 and hence induced violet-red pigment (in Parental strain cells), the lag period of growth was twice as long (approximately 60 min, Figure 13 c,d). Therefore, the presence of pigment in Parental strain cells adversely affected the response of the cell to growth stimuli.

Parental Cultures

The growth curves for Parental strain cultures grown for 6 hours before irradiation in the absence of FeCl_3 , were characterized by a period of rapid growth, followed by some cell death and subsequent renewed growth at the same rate as in the control cultures (Figure 12). The duration of the lag period prior to the onset of rapid growth was found to be dependent on both the pre- and the post-irradiation media. That is to say, the presence of FeCl_3 in the medium, either before or after irradiation, delayed the onset of the rapid growth period for about 60 minutes (Figure 12 b, c). When FeCl_3 was absent no lag was detected (Figure 12 a), but when iron was present in both pre- and post-irradiation media, the lag period was about 90 minutes (Figure 12 d). No cell death was detected during the lag phase.

The duration of the rapid growth period was found to be twice as long, when FeCl_3 was present prior to irradiation. In the absence of FeCl_3 this period lasted 30 to 40 minutes (Figure 12 a, b), but in the presence of FeCl_3 this period extended to approximately 60 minutes (Figure 12 c, d). The slope of the viability curve during this period was found to be constant for all the cultures.

The period of decline (probably due to delayed cell death) appears to be dependent on the presence of FeCl_3 in the post-irradiation growth medium and totally independent of the presence of this compound in the pre-irradiation

medium (Figure 12). The cell cultures exposed to FeCl_3 after irradiation declined in growth rate for about 30 min, while the cultures lacking FeCl_3 in the post-irradiation medium declined for 50 or more minutes (Figure 12).

Figure 12.

Parental cells grown 6 hours before irradiation.

(a)

Pre-irradiation medium --- Basal
Post-irradiation medium --- Basal

Plate Medium

○ ——— ○ - control - Basal
△ ——— △ - control - Basal + FeCl₃
● ——— ● - test - Basal
▲ ——— ▲ - test - Basal + FeCl₃

(b)

Pre-irradiation medium ---- Basal
Post-irradiation medium --- Basal + FeCl₃

Plate Medium

○ ——— ○ - control - Basal
□ ——— □ - control - Basal + FeCl₃
● ——— ● - test - Basal
■ ——— ■ - test - Basal + FeCl₃

(c)

Pre-irradiation medium ---- Basal + FeCl₃
Post-irradiation medium --- Basal

Plate Medium

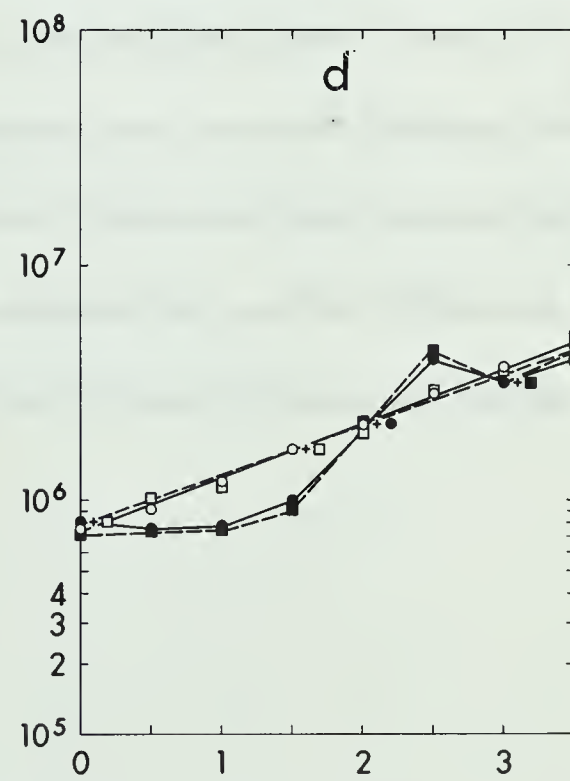
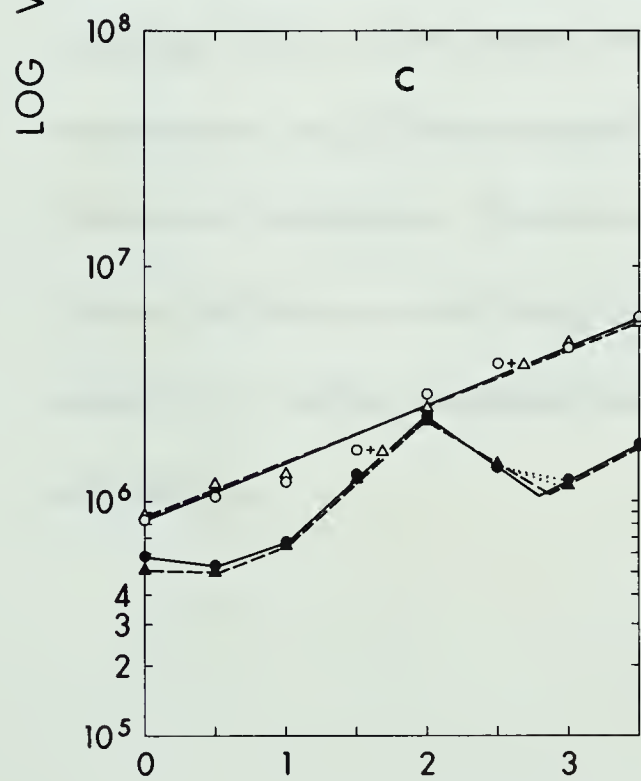
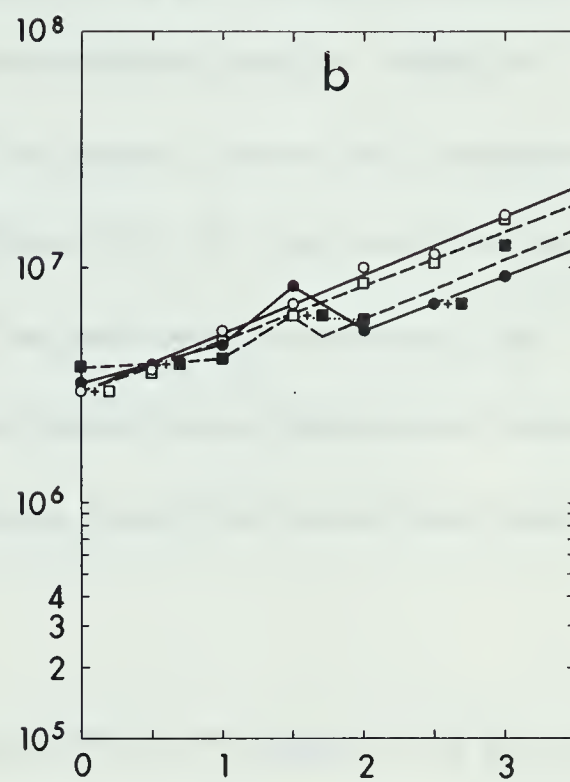
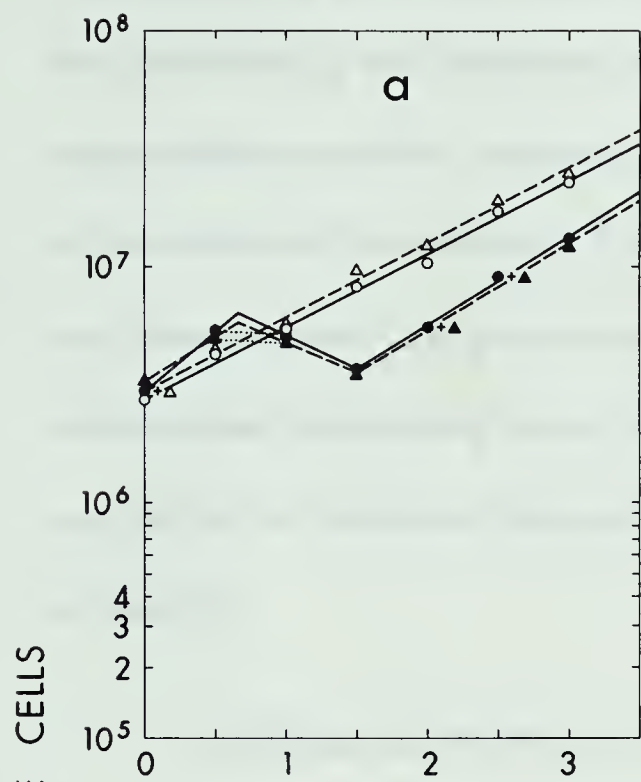
○ ——— ○ - control - Basal
△ ——— △ - control - Basal + FeCl₃
● ——— ● - test - Basal
▲ ——— ▲ - test - Basal + FeCl₃

(d)

Pre-irradiation medium ---- Basal + Fe
Post-irradiation medium --- Basal + FeCl₃

Plate Medium

○ ——— ○ - control - Basal
□ ——— □ - control - Basal + FeCl₃
● ——— ● - test - Basal
■ ——— ■ - test - Basal + FeCl₃



The growth of 16 hour test cultures of the Parental strain was influenced by the presence or absence of FeCl_3 in the post-irradiation medium but unaffected by this difference in the pre-irradiation medium. When the post-irradiation medium contained FeCl_3 , the growth curve of cells appeared to be unaffected by radiation (γ -rays) (Figure 13 b, d). No detectable lag was observed when FeCl_3 was absent from the post-irradiation medium; the cells entered immediately into a logarithmic growth phase (the slope of which was similar to the slope of the control cultures during their logarithmic phase of growth).

Different sensitivities to ionizing radiation of bacterial cells (E. coli) during the various stages of the growth cycle was reported by Stapleton (1955, (see page 20)). In the present work, the cells irradiated after 6 hours of incubation are in a different stage of the growth cycle than cells irradiated after 16 hours of incubation. The 6 hour cells are in the logarithmic phase while the 16 hour cells are in a stationary phase. Because of the possible differences in sensitivity, a valid comparison between 6 and 16 hour cultures could not be undertaken.

Figure 13.

Parental cells grown 16 hours before irradiation.

(a)

Pre-irradiation medium ---- Basal
Post-irradiation medium --- Basal

Plate Medium

○ ——— ○ - control - Basal
△ ——— △ - control - Basal + FeCl₃
● ——— ● - test - Basal
▲ ——— ▲ - test - Basal + FeCl₃

(b)

Pre-irradiation medium ---- Basal
Post-irradiation medium --- Basal + FeCl₃

Plate Medium

○ ——— ○ - control - Basal
□ ——— □ - control - Basal + FeCl₃
● ——— ● - test - Basal
■ ——— ■ - test - Basal + FeCl₃

(c)

Pre-irradiation medium ---- Basal + FeCl₃
Post-irradiation medium --- Basal

Plate Medium

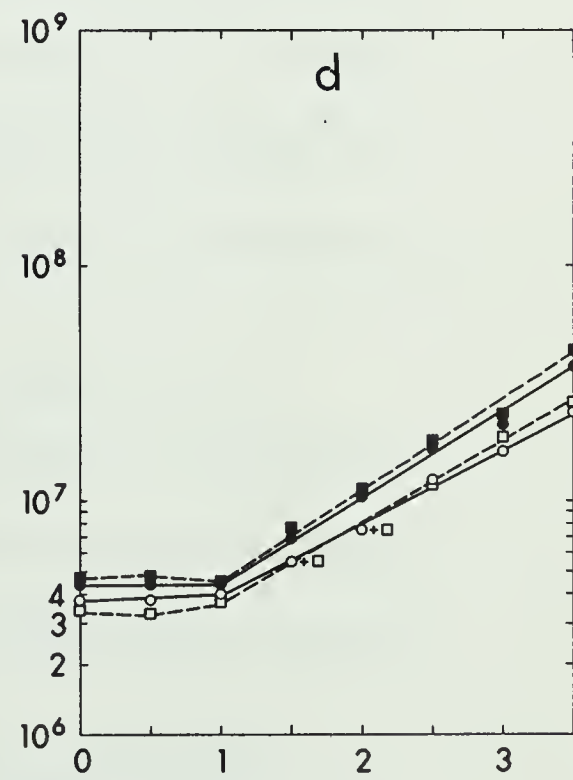
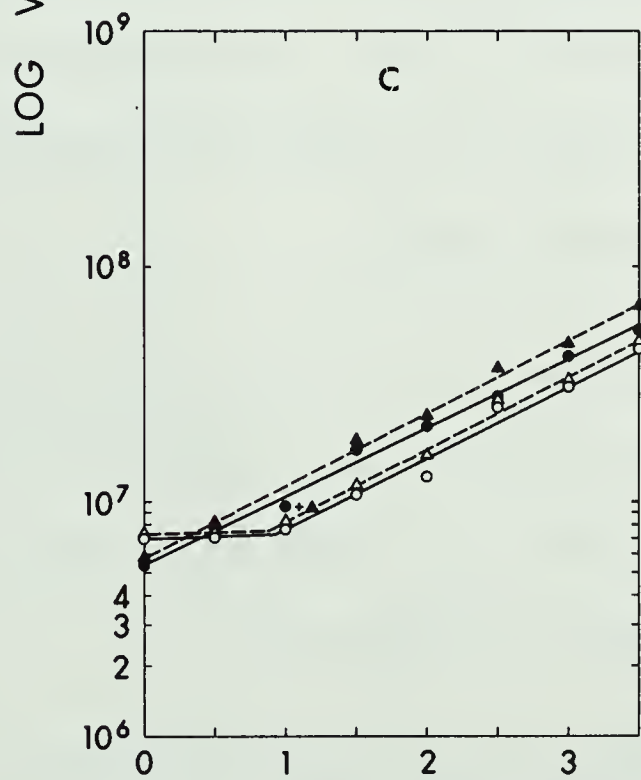
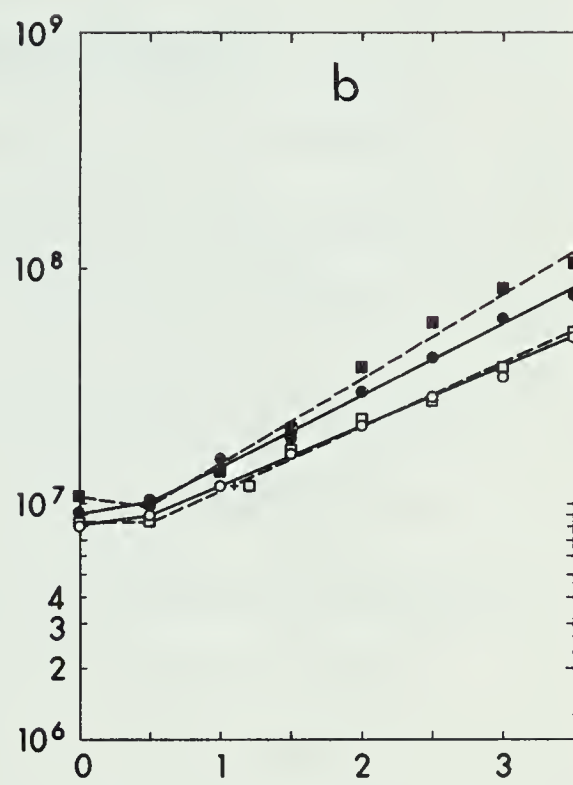
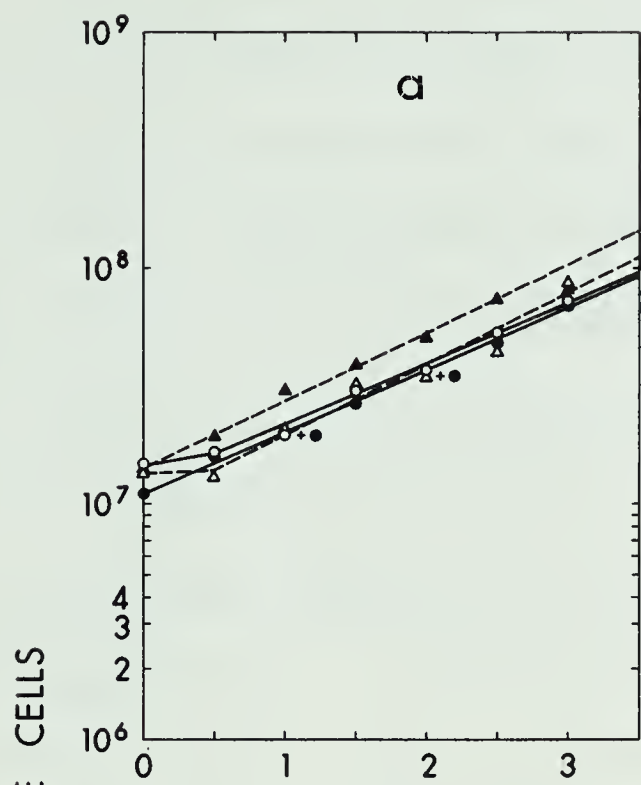
○ ——— ○ - control - Basal
△ ——— △ - control - Basal + FeCl₃
● ——— ● - test - Basal
▲ ——— ▲ - test - Basal + FeCl₃

(d)

Pre-irradiation medium ---- Basal + Fe
Post-irradiation medium --- Basal + FeCl₃

Plate Medium

○ ——— ○ - control - Basal
□ ——— □ - control - Basal + FeCl₃
● ——— ● - test - Basal
■ ——— ■ - test - Basal + FeCl₃



Superpig Cultures

No lag period was evident in either the 6 hour or the 16 hour Superpig control cultures. The cells started to divide as soon as they were transferred to fresh medium. The cells of the 16 hour cultures divided at a much faster rate on transfer than cells of the 6 hour cultures (doubling time for 6 hour culture amounted to approximately 90 min, while the doubling time for the 16 hour culture was about 55 min, Figure 14, 15). It appears that whilst pigment production is active (i.e., the cell is in the stationary phase of the growth cycle), the presence of new growth stimulants (i.e., fresh medium) induces the cells to divide at a very rapid rate. It is clear, that not all cells in a stationary culture have ceased dividing; as cells die, other cells reproduce to maintain the stationary appearance of the culture. When Superpig cells in the stationary phase of growth encounter fresh medium, they respond rapidly which manifests itself by the initiation of division.

All the Superpig cultures grown for 6 hours before being irradiated exhibited long lag periods with little or no cell death, followed by a lengthy rapid growth phase and a lengthy decline phase. Following the decline, the cells grew at equal rates as the control cells (Figure 14).

The lag phase, which lasted from 30 to 60 min, appears to be the time necessary for cells to adapt to replenished medium. During this phase no, or only a slight reduction in the viable count was observed.

The lag period was followed by the rapid growth phase, which lasted from 60 to 90 min, depending on whether or not the fresh medium was the same as the original medium. When the fresh medium was different, the rapid growth phase lasted for about 60 min (Figure 14 b, c). However, post-irradiation medium of the same composition as the pre-irradiation medium, caused this phase to last up to 90 minutes (Figure 14 a, d). The period of decline was lowest when FeCl_3 was absent from both the pre- and the post-irradiation media. Differences in the duration of this period could not be attributed to the changes in the environments encountered by the Superpig cells.

Figure 14.

Superpig cells grown 6 hours before irradiation.

(a)

Pre-irradiation medium ---- Basal
Post-irradiation medium --- Basal

Plate Medium

○ ——— ○ - control - Basal
△ ——— △ - control - Basal + FeCl₃
● ——— ● - test - Basal
▲ ——— ▲ - test - Basal + FeCl₃

(b)

Pre-irradiation medium ---- Basal
Post-irradiation medium --- Basal + FeCl₃

Plate Medium

○ ——— ○ - control - Basal
□ ——— □ - control - Basal + FeCl₃
● ——— ● - test - Basal
■ ——— ■ - test - Basal + FeCl₃

(c)

Pre-irradiation medium ---- Basal + FeCl₃
Post-irradiation medium --- Basal

Plate Medium

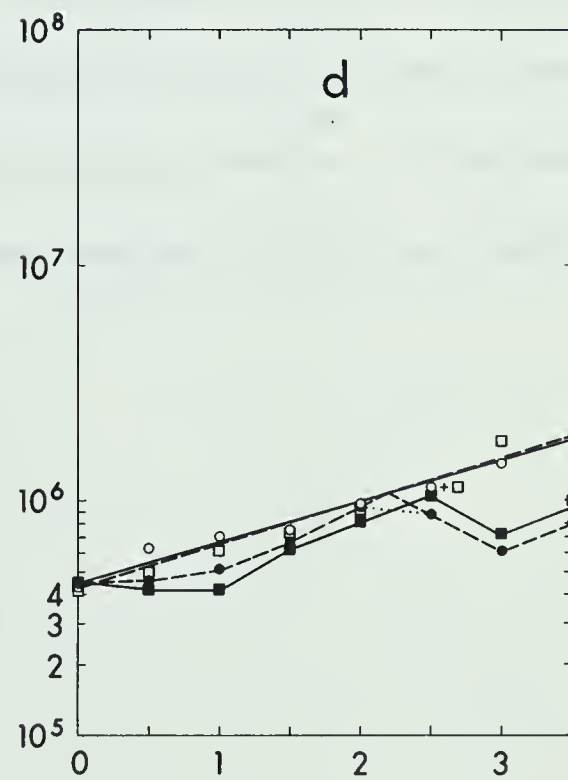
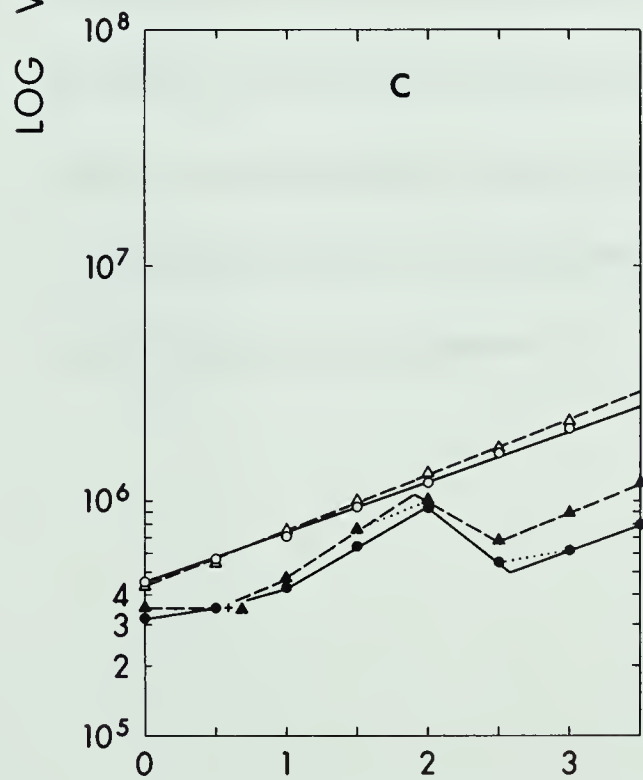
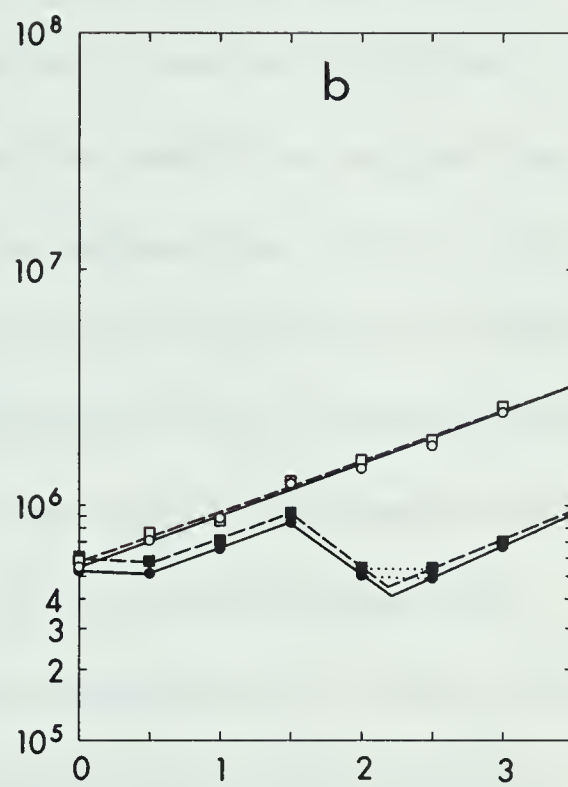
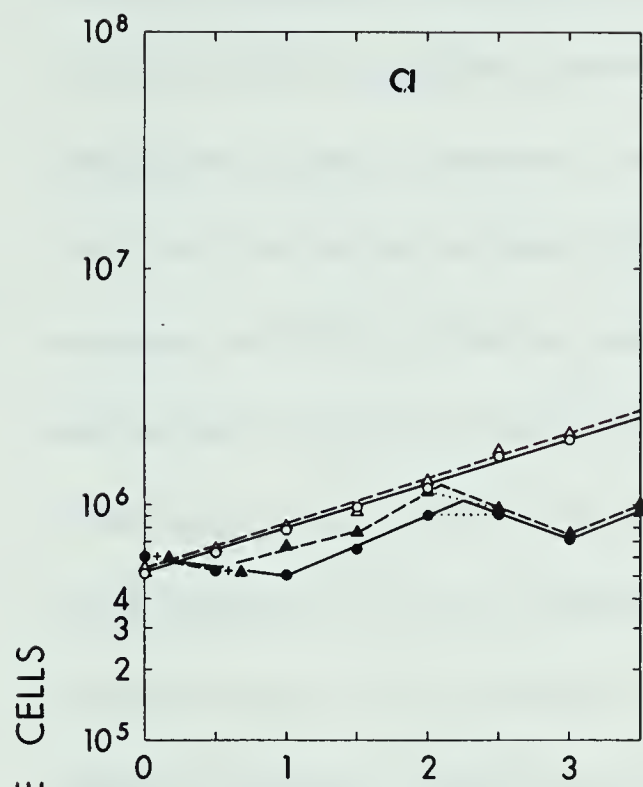
○ ——— ○ - control - Basal
△ ——— △ - control - Basal + FeCl₃
● ——— ● - test - Basal
▲ ——— ▲ - test - Basal + FeCl₃

(d)

Pre-irradiation medium ---- Basal + Fe
Post-irradiation medium --- Basal + FeCl₃

Plate Medium

○ ——— ○ - control - Basal
□ ——— □ - control - Basal + FeCl₃
● ——— ● - test - Basal
■ ——— ■ - test - Basal + FeCl₃



POST - IRRADIATION INCUBATION TIME (hr)

Cultures of Superpig cells radiated after 16 hours of growth and following irradiation, immediately transferred to fresh medium were characterized by their logarithmic growth. Transfer to a medium with a different composition caused a decrease of the slope of the viability curve, when compared with the respective slope of the control cultures (Figure 15b, c). For example, the doubling time for control culture cells originally grown in basal medium and transferred to medium supplemented with FeCl_3 , was approximately 55 minutes, whereas cells raised under the same conditions but irradiated before being transferred, showed a doubling time of 70 - 90 minutes (Figure 15b). Cells transferred to fresh medium of the same composition as the initial medium, showed an identical viability curve slope (and therefore identical doubling time) when compared with control culture cells (Figure 15 a, d). The presence of FeCl_3 in the medium before and after radiation (Figure 15d) caused a short 30 minute lag period on transfer to the post-irradiation medium. In the absence of FeCl_3 from the pre- and/or post-irradiation medium, no lag in the viable cell count curve was observed.

Figure 15.

Superpig cells grown 16 hours before irradiation.

(a)

Pre-irradiation medium ---- Basal
Post-irradiation medium --- Basal

Plate Medium

○ ——— ○ - control - Basal
△ ——— △ - control - Basal + FeCl₃
● ——— ● - test - Basal
▲ ——— ▲ - test - Basal + FeCl₃

(b)

Pre-irradiation medium ---- Basal
Post-irradiation medium --- Basal + FeCl₃

Plate Medium

○ ——— ○ - control - Basal
□ ——— □ - control - Basal + FeCl₃
● ——— ● - test - Basal
■ ——— ■ - test - Basal + FeCl₃

(c)

Pre-irradiation medium ---- Basal + FeCl₃
Post-irradiation medium --- Basal

Plate Medium

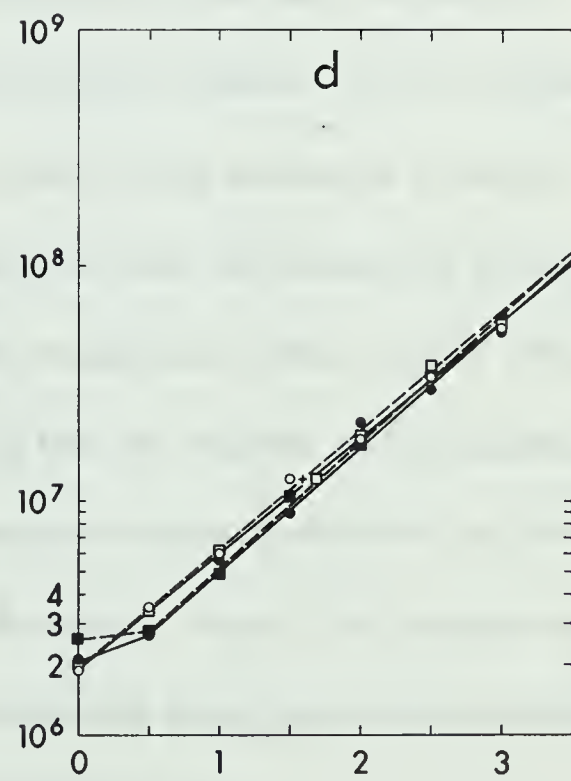
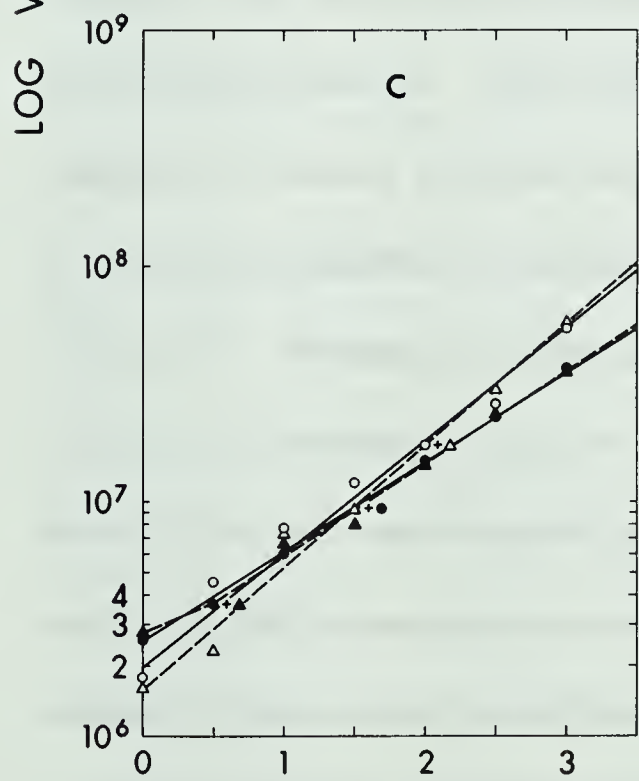
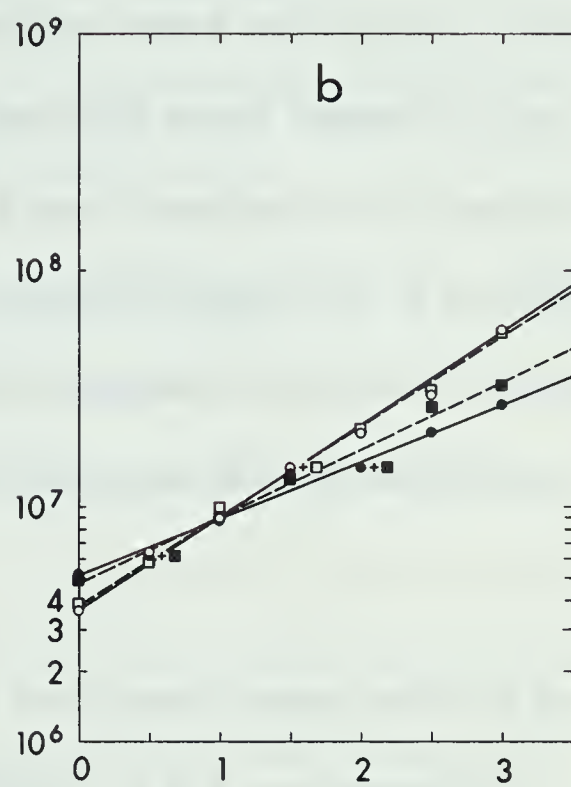
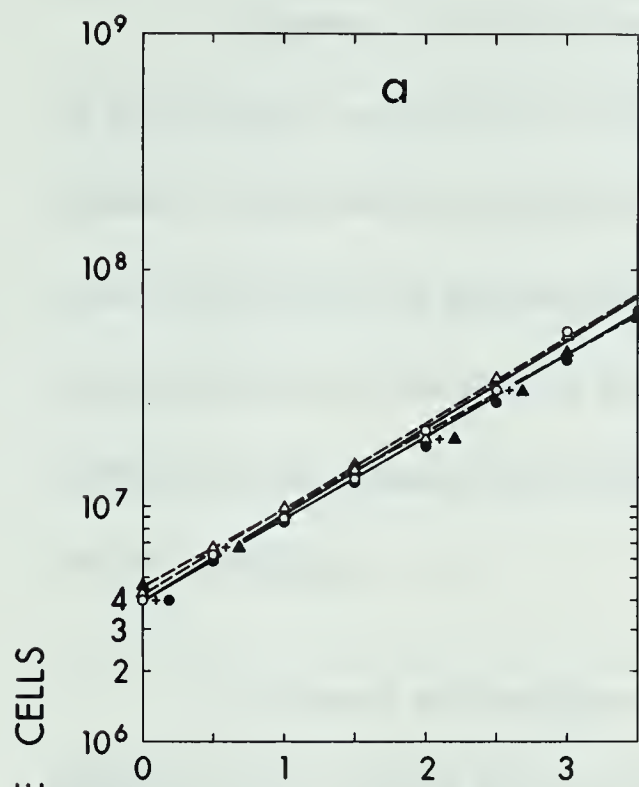
○ ——— ○ - control - Basal
△ ——— △ - control - Basal + FeCl₃
● ——— ● - test - Basal
▲ ——— ▲ - test - Basal + FeCl₃

(d)

Pre-irradiation medium ---- Basal + Fe
Post-irradiation medium --- Basal + FeCl₃

Plate Medium

○ ——— ○ - control - Basal
□ ——— □ - control - Basal + FeCl₃
● ——— ● - test - Basal
■ ——— ■ - test - Basal + FeCl₃



POST - IRRADIATION INCUBATION TIME (hr)

Littlepig Cultures

In general, survival curves of Littlepig control cultures grown initially for 6 hours were very similar to those obtained with control Parental cultures. Transfer to fresh medium caused the cells to enter immediately into logarithmic growth and little or no lag period could be detected (Figure 16). It is evident, that the cells were in a state of division before transfer and continued to divide in their new environment with little, or no detectable delay for adjustment to the new condition.

The control cultures grown for 16 hours before transfer exhibited lag periods in their viability count which lasted for 1/2 to 1 hour depending on whether or not the cells were transferred to medium of the same composition as the original medium. Cells transferred to medium different from the original, required a 30 minute period for adjustment, while cells transferred to medium of the same composition as the original, required about 60 minutes for adjustment (Figure 17). Stationary Littlepig cells when transferred to fresh medium different (either by the presence or absence of FeCl_3) from the original medium adjusted faster to their new environmental conditions than cultures transferred to a fresh medium of similar composition as the initial medium. Hence, the presence or absence of one or more factors (iron ions or chloride ions) appears to be sufficient to cause a stimulation of cell growth inducing factors.

Littlepig cells radiated for 1 minute after 6 hours of growth, exhibited

a growth lag period characterized by cell death. Following the lag period, the cells entered into a state of rapid division and subsequently into a second period of cell death. On termination of the second period, cell divisions occurred in the same rate as in control cultures (Figure 16).

The initial period of cell death lasted for about 60 minutes, except when the pre-irradiation medium consisted of basal medium and the post-irradiation medium was supplemented with FeCl_3 . In the latter case the initial period of cell death lasted for 30 minutes. The rate of death varied considerably depending on the pre- and post-irradiation media. When FeCl_3 was absent from both the pre- and post-irradiation media the viability count was reduced by about $1/7$, after 1 hour of incubation (Figure 16 a), but when FeCl_3 was present in the post-irradiation medium the reduction amounted to $3/8$ of the original count after 30 min of incubation (Figure 16 b). It appears that the presence of FeCl_3 in the medium after exposure of the cells to radiation inhibits their recovery. When, on the other hand, FeCl_3 was present in the pre-irradiation medium, the effect of its presence in the post-irradiation medium was much reduced. For example, the viability count decreased by $1/3$ in the absence of FeCl_3 from the post-irradiation medium and by $2/5$ in the presence of FeCl_3 (Figure 16 c, d).

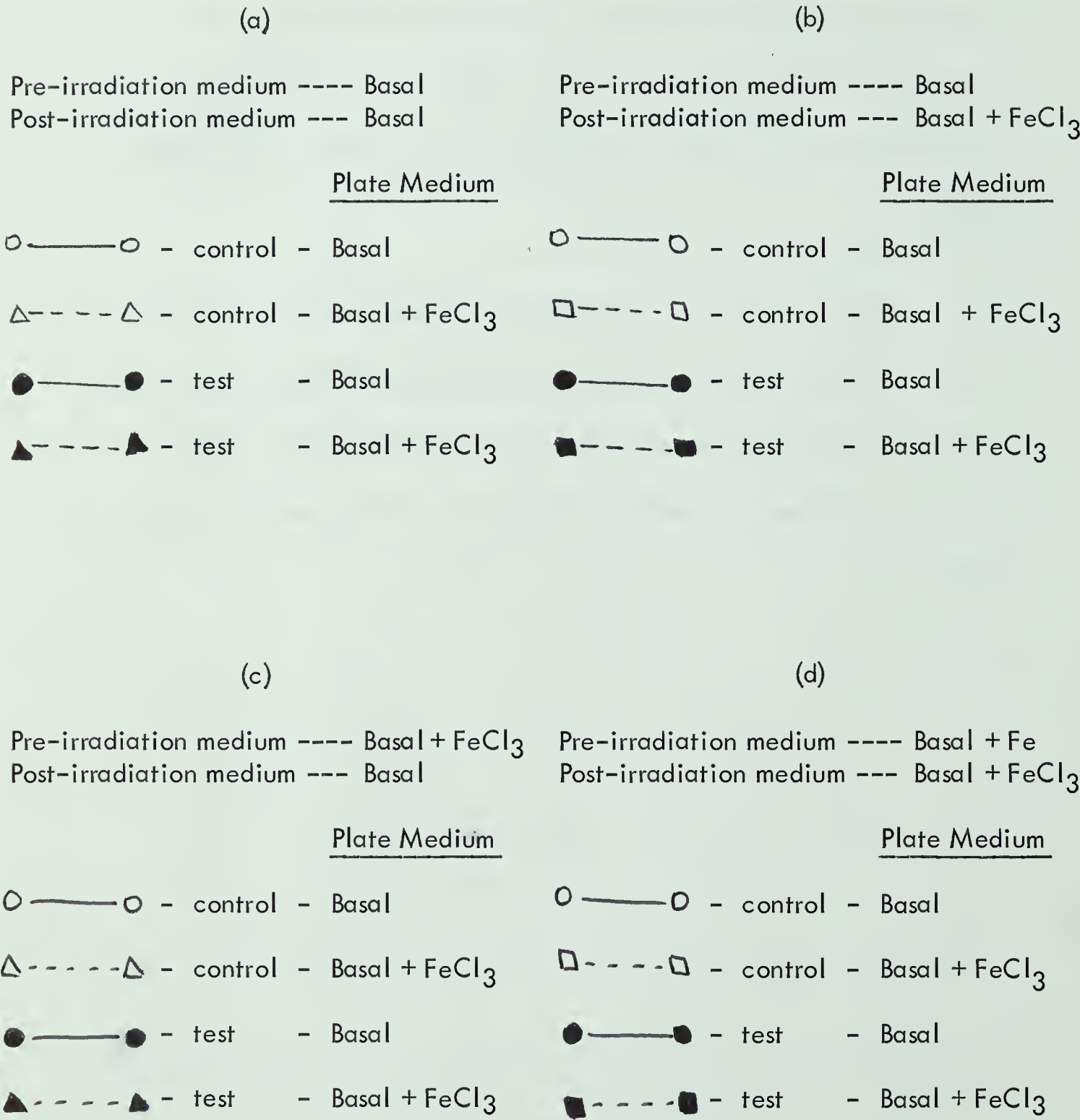
The duration of the rapid growth phase was found to be dependent on the post-irradiation medium. The absence of FeCl_3 from the medium caused this phase to last for approximately 45 minutes. Although in Figures 16 a and 16 c the growth phase maxima were extrapolated, results of different plating

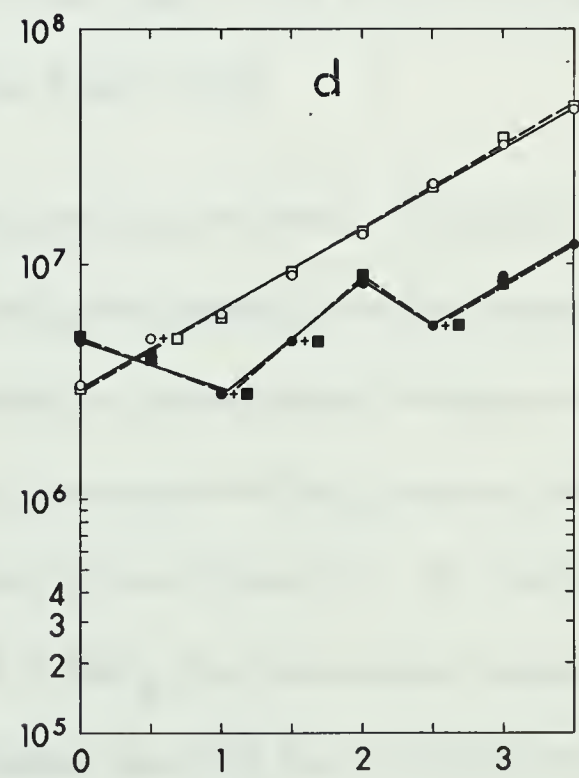
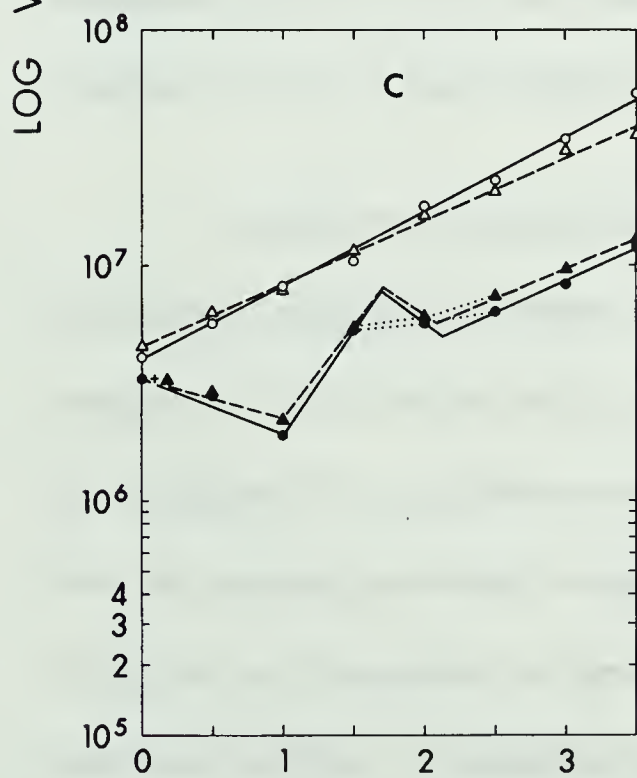
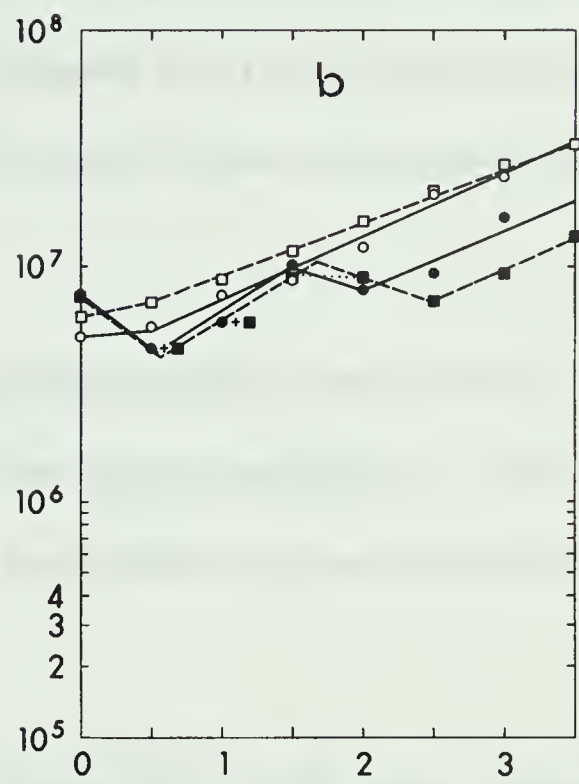
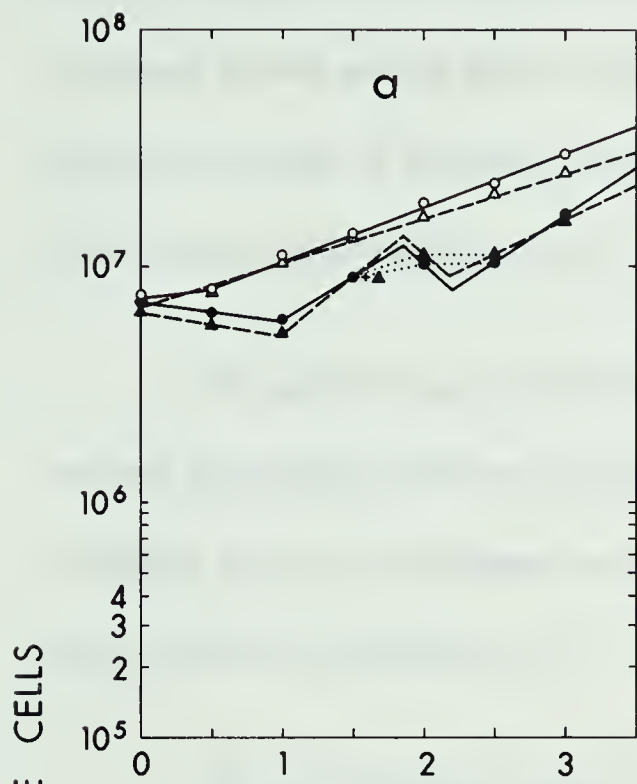
times (15, 45 and 75 min) indicate that the calculated maxima coincide with the experimental ones. The presence of FeCl_3 in the post-irradiation medium increased the rate of growth of the organism, however the magnitude of the increase was dependent on whether or not the pre-irradiation medium was supplemented with FeCl_3 (Figure 16 b, d). Cells exposed to FeCl_3 prior to irradiation divided at a much faster rate than cells grown initially in the absence of FeCl_3 .

The second period of cell decline was found to be very short (approximately 30 - 45 min) and independent (in duration) of the presence or absence of FeCl_3 in the pre- or post-irradiation media. The rate of decline was similar under all conditions and therefore independent of the presence or absence of FeCl_3 .

Figure 16.

Littlepig cells grown 6 hours before irradiation.





POST - IRRADIATION INCUBATION TIME (hr)

Viability curves of Littlepig cells grown for 16 hours prior to irradiation were similar to those obtained with cells after 6 hours of initial incubation. Increased growth periods after a short lag, followed by a period of decline and subsequent growth at the same rate as in the control cultures, were characteristic for all curves obtained (Figure 17).

The growth lag period was of short duration (approximately 30 min) and was found to be uniform throughout all the littlepig test samples. During this latter phase no significant decrease in the viability count was observed in any of the cultures (Figure 17).

The rapid division phase lasted for about 30 min in all cases. However, a maximum rate of division was obtained in those cases, where FeCl_3 was absent in either the pre- or post-irradiation medium (Figure 17 a).

A period of cell death followed the rapid growth phase. The duration of this phase depended on whether or not FeCl_3 was present in the pre-irradiation medium. In media lacking FeCl_3 , the viability count decreased for approximately 30 min (Figure 17 a, b), while in media supplemented with FeCl_3 prior to irradiation, the decrease amounted to approximately 45 min (Figure 17 c, d). The rate of decline was independent of the presence of FeCl_3 in either the pre- or post-irradiation medium. A logarithmic growth phase (in which the cells of the test cultures divided at the same rate as their controls) followed the period of decline.

Figure 17.

Littlepig cells grown 16 hours before irradiation.

(a)

Pre-irradiation medium ---- Basal
Post-irradiation medium --- Basal

Plate Medium

○ ——— ○ - control - Basal
△ ——— △ - control - Basal + FeCl₃
● ——— ● - test - Basal
▲ ——— ▲ - test - Basal + FeCl₃

(b)

Pre-irradiation medium ---- Basal
Post-irradiation medium --- Basal + FeCl₃

Plate Medium

○ ——— ○ - control - Basal
□ ——— □ - control - Basal + FeCl₃
● ——— ● - test - Basal
■ ——— ■ - test - Basal + FeCl₃

(c)

Pre-irradiation medium ---- Basal + FeCl₃
Post-irradiation medium --- Basal

Plate Medium

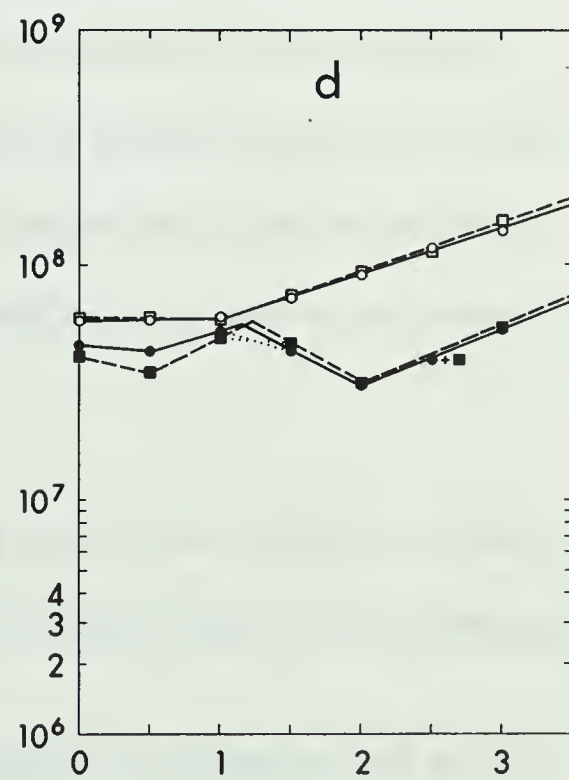
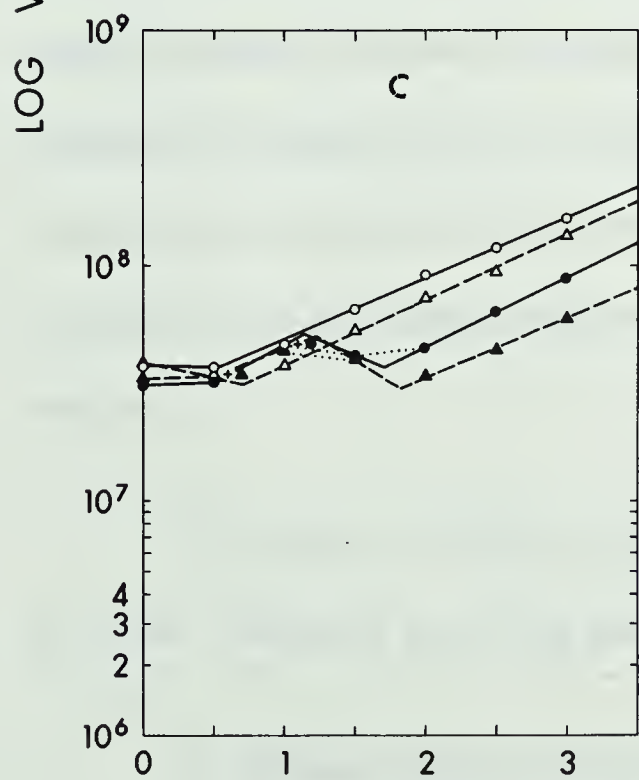
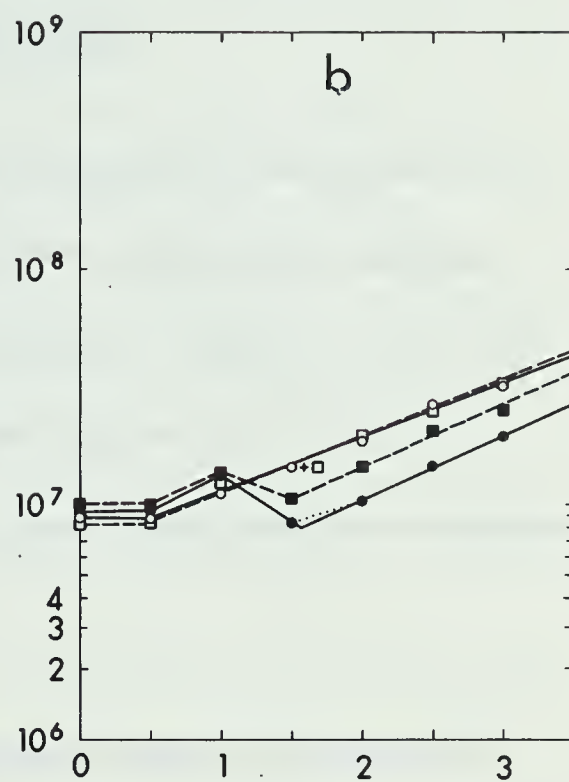
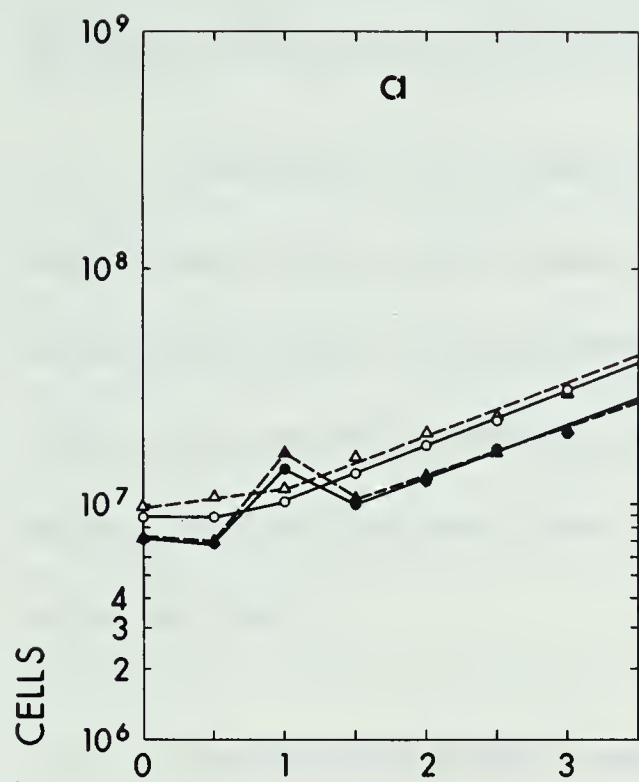
○ ——— ○ - control - Basal
△ ——— △ - control - Basal + FeCl₃
● ——— ● - test - Basal
▲ ——— ▲ - test - Basal + FeCl₃

(d)

Pre-irradiation medium ---- Basal + Fe
Post-irradiation medium --- Basal + FeCl₃

Plate Medium

○ ——— ○ - control - Basal
□ ——— □ - control - Basal + FeCl₃
● ——— ● - test - Basal
■ ——— ■ - test - Basal + FeCl₃



Comparison of the effects of γ -radiation on 6 hour and 16 hour cultures of Parental - , Superpig - and Littlepig strain cultures.

Examination of the viability curves for control cultures grown for 6 hours before transfer to fresh medium, showed that under similar conditions all three strains grew at approximately the same rate. The slopes of the viability curves were all found to be quite similar (Figures 12, 14, 16). In addition, the doubling time of the viability count of the three strains employed varied very little.

Similar growth patterns were encountered when the three genetic clones were irradiated after 6 hours of growth. All strains exhibited a lag period in growth, followed by a period of rapid growth, which in turn was followed by a period of decline, and finally by a period of growth at the same rate as the control. The length of each of these periods varied depending on the strain, or on the absence or presence of FeCl_3 in the pre- or post-irradiation medium.

The presence of FeCl_3 in either the pre- or post-irradiation medium, or in both, induced a growth lag period in the Parental strain cultures (Figure 12).

In Littlepig strain cultures the lag period was characterized by cell death and was present regardless of the FeCl_3 content of the medium (Figure 16). The amount of cell death was affected by the presence of FeCl_3 in either the pre-

or post-irradiation medium, or in both. Lengthy lag periods with little or no cell death characterized the 6 hour Superpig cultures (Figure 14).

The presence of FeCl_3 in the pre-irradiation medium caused the rapid growth period of the Parental strain cells to last twice as long as when no FeCl_3 was present. Duplication time during this period was nearly constant for all strains employed, varying from 35 - 45 minutes (Figure 12). For Littlepig strain cells the length of the rapid growth period was dependent on the composition of the post-irradiation medium. When FeCl_3 was absent from this medium the rapid growth period lasted 45 min, but when FeCl_3 was present, it extended to 60 min (Figure 16). Cells exposed to FeCl_3 before radiation, divided at a much faster rate than cells which were not exposed to FeCl_3 . For example, cell duplication time amounted to 50 min in the absence of FeCl_3 before irradiation. However, when FeCl_3 was present cell duplication time was reduced to 25 - 35 min. The length of the rapid growth phase of the Superpig cells was dependent on whether or not the cells were grown in the same medium before and after irradiation. When a change was instituted, this phase lasted for 60 min, but without a change a phase as long as 90 min was found (Figure 14). Duplication time was about the same under all conditions. It varied from 60 - 75 min.

The period of rapid decline in the Parental strain cells depended on the presence or absence of FeCl_3 in the post-irradiation medium. FeCl_3 was present when this period lasted 30 min or less, but in the absence of FeCl_3 the

period extended to approximately 60 min (Figure 12). In Littlepig and Superpig strain cells the presence or absence of FeCl_3 had no effect on the 30 - 50 min duration of the decline period (Figure 14, 16). A minimum rate of viability decline was obtained for Littlepig strain cells when the treatment consisted of a basal pre-irradiation medium but a FeCl_3 supplemented post-irradiation medium. For Superpig strain cells a minimum rate was obtained when grown in a basal (i.e. no FeCl_3) pre- as well as post-irradiation medium.

Parental strain 16 hour control cultures showed a growth lag period which lasted for approximately 30 min when cells were grown in the absence of FeCl_3 before transference to a fresh medium. A lag of approximately 60 min was found when the culture medium (i.e., before transfer of the cells) contained FeCl_3 . In Parental strain control cultures (Figure 13), the presence or absence of FeCl_3 in the fresh medium had no effect on the duration of the lag period. Littlepig strain control cultures were also characterized by a lag period followed by logarithmic growth. The duration of the lag was dependent on whether or not the cells were transferred to fresh medium which differed in composition from the initial medium.

The presence of the growth lag period in the Parental strain 16 hour test cultures (irradiated for 1 min, 12 krads) depended on the presence of FeCl_3 in the post-irradiation medium. That is to say, when FeCl_3 was present, a lag period equal in duration to that of the control, was observed. No lag period was observed in the absence of FeCl_3 from the post-irradiation medium (Figure 13).

The growth of irradiated cultures of the 16 hour Superpig strain appeared to be almost unaffected by the γ -radiation itself. When compared with the controls, logarithmic growth was much slower for cells grown in a medium after transfer, which differed in composition (i.e., by the presence or absence of FeCl_3) from the initial medium. When the transfer was to the same medium, the logarithmic growth was the same. A 30 min growth lag period was present when both, the pre- and post-irradiation media, contained FeCl_3 (Figure 15).

The irradiated Littlepig 16 hour cultures were found to be unique among the 16 hour cultures. A short 30 min rapid growth phase was detected after a 30 min growth lag period in which there was no cell death. Following the rapid growth phase, a period of cell death (e.g., a decline of the viability count) was observed. This period of cell death was followed by a logarithmic growth phase similar to that of control cultures (Figure 17). For transfer to a different medium the lag period was found to last approximately 30 min, whereas for an identical subsequent medium the lag period was about twice as long (approximately 60 min, Figure 17). No lag period was evident in Superpig strain 16 hour control cultures (Figure 15). The latter cells initiated logarithmic growth immediately.

A comparison of the doubling times during the logarithmic phase of growth of the three strains employed showed, that the Superpig strain cells divided relatively fast (40 - 50 min doubling time), followed by the Parental

strain cells (60 - 70 min doubling time) and the Littlepig strain cells (75 - 90 min doubling time).

Initial cell divisions with a shorter generation time occurring after exposure to ionizing radiation have been reported for E. coli B by Alper (1957) and by Laser and Thornley (1957). Alper found that a dose of 3 krads delivered to the cell suspension at the end of the normal lag phase (cells have considerably less sensitivity to ionizing radiation than cells in the resting state (see page 20)) causes a longer lag phase than obtained in control experiments. However on initiation of the division the cell generation time for approximately four generations decreased by a factor of two when compared with normal bacteria. Laser and Thornley on the other hand, found that a change in the nutrients of the post-irradiation medium was a prerequisite for a decrease in cell division time.

SUMMARY AND CONCLUSIONS

1. The presence of FeCl_3 in the medium induces the production of the iron containing pigment pulcherrimin, in the Parental and Superpig strain cells, and an iron free pigment precursor in the Littlepig strain cells.
2. The protective effect of FeCl_3 as an additive to the pre-irradiation medium causes an increase of approximately 100% in the D_{37} for all three strains.
3. The availability of ferric ions in the post-irradiation medium gives rise to a substantial increase of the D_{37} , indicating that the ferric ion is important for the repair of radiation damage.
4. The radioprotective action of FeCl_3 on cells of M. violagabriellae may be brought about by at least two mechanisms:
 - (i) ferric ions act as free radical scavengers,
 - (ii) ferric ions by means of charge neutralization prevent excessive loss of cell constituents caused by the radiation-induced increase in cell permeability.
5. The removal of radioactively labelled ferric ions (^{59}Fe) from the medium by the three strains of M. violagabriellae correlated positive with the amount of pigment produced by the three strains.

6. Some sort of cellular regulatory mechanism is operative in the control of pigment synthesis in the Parental-strain cells. The Littlepig-strain cells have an alteration in their genetic material, the result of which is the prevention of the incorporation of iron into the pigment molecule. The Superpig strain cells have an alteration in their genetic material which affects the control of pigment synthesis.

7. Identical post-irradiation growth patterns were encountered when the three genetic clones were irradiated after 6 hours of growth. All strains exhibited a lag period in growth, followed by a period of rapid growth, which in turn was followed by a period of decline, and finally by a period of growth at the same rate as the control. The length of each of these periods varied depending on the strain, or on the absence or presence of FeCl_3 in the pre- or post-irradiation medium.

8. Different growth patterns were obtained for the three genetic strains when irradiation was carried out after 16 hours of initial growth. A growth pattern identical to the one observed for all three irradiated strains (see Sub.7) after 6 hours of initial growth, was found to occur in Littlepig strain cells, Superpig and Parental strain cultures appeared to be unaffected by the γ -rays after 16 hours of pre-irradiation incubation.

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